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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68, G01N 33/53 — A2

(11) International Publication Number: **WO 00/29624**

(43) International Publication Date: 25 May 2000 (25.05.00)

(21) International Application Number: PCT/US99/27525

(22) International Filing Date: 19 November 1999 (19.11.99)

(30) Priority Data:

60/109,082	19 November 1998 (19.11.98)	US
60/117,389	27 January 1999 (27.01.99)	US
60/132,976	7 May 1999 (07.05.99)	US
Not furnished	3 November 1999 (03.11.99)	US
Not furnished	3 November 1999 (03.11.99)	US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

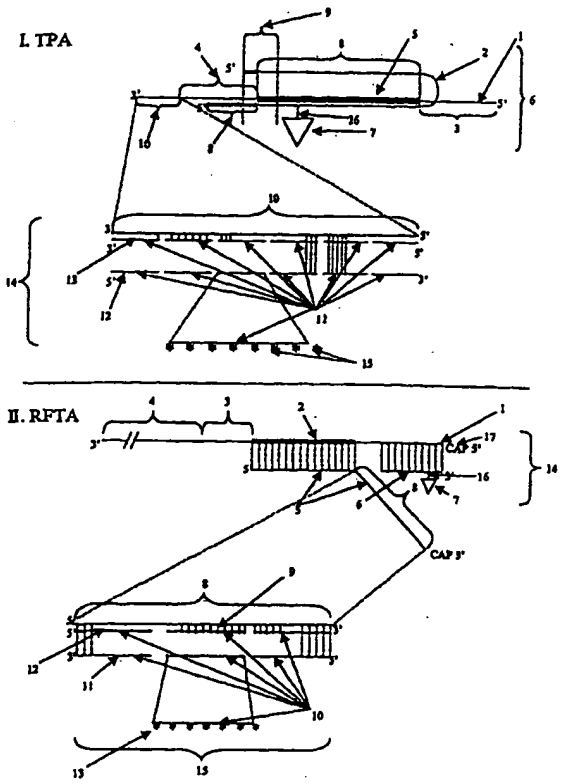
Published

Without international search report and to be republished upon receipt of that report.

(54) Title: METHODS AND COMPOSITIONS FOR DETECTION OF NUCLEIC ACID SEQUENCES AND SIGNAL AMPLIFICATION

(57) Abstract

The present invention comprises compositions and methods directed to analysis and detection of target nucleic acid sequences. Particularly, the methods and compositions comprise formation of duplex and triplex structures. In some embodiments, hairpin structures are employed. Novel restriction methods are provided for restriction of nucleic acids. Signal amplification systems, including duplex, triplex and MTRF systems, are also provided that allow for detection of very low copy number targets. These compositions and methods are especially useful in diagnostic assays.



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METHODS AND COMPOSITIONS FOR DETECTION OF NUCLEIC ACID SEQUENCES AND SIGNAL AMPLIFICATION

Cross-Reference to Related Application

This application claims priority to U.S. Provisional Patent Application No. 60/109,082 filed November 19, 1998, U.S. Provisional Patent Application No. 60/117,389 filed January 27, 1999, U.S. Provisional Patent Application No. 60/132,976 filed May 7, 1999, U.S. Provisional Patent Application No. 60/_____ filed November 3, 1999, atty no. 03038-0190P, and U.S. Provisional Patent Application No. 60/_____ filed November 3, 1999, atty no. 03038-0191P, all of which are herein incorporated in their entirety.

Background of the Invention

Advances in molecular biological techniques have provided a large repertoire of procedures for the identifying and quantifying of specific DNA and RNA sequences. It was quickly recognized that such methods could be exploited as powerful elements of diagnostic tests for the detection of microbial or genetic pathologies, or genetic variation. Traditional serologically-based protocols often do not achieve their anticipated results. Cross-reactivity frequently presents insurmountable obstacles. Furthermore, there is often a significant time lag, sometimes extending to weeks or months, before an immune response attains detectable levels, precluding the early detection of infections that are best treated before irreparable damage or death occurs. However, despite the advantages offered by DNA and RNA-based technology to quickly and accurately detect specific nucleic acid sequences, and the proliferation of diagnostic tests based thereon, even these procedures have demonstrable inherent limitations, that together restrict their value as practical diagnostic tools in the medical environment.

RNA and DNA-based diagnostic technology must be capable of the early detection of infectious disease, cancer, genetic hereditary testing, and offer high sensitivity and specificity, time and cost efficiency, and a simplistic process design that is amenable to automation for high-volume routine use. However, procedures designed to analyze DNA or RNA, have common fundamental features and share many of the same faults. Thus, since targeted nucleic acid sequences are usually available at low levels, of necessity, most procedures incorporate elements that amplify either the target nucleic acid sequence itself, or the signal marking that the target is present in the test sample.

Many target and signal amplification methods are described in the literature, but none offer the optimum combination of high specificity, thus detecting only the intended target sequence, simplicity of operation, and speed in reaching a result, all factors crucial to a practical diagnostic test. Among these procedures are found the target amplification methods of nucleic acid sequence-based amplification (NASBA Organon Teknika), strand-displacement amplification (Becton Dickinson), transcription-based amplification system (TAS), transcription-mediated amplification (Genprobe), and above all the polymerase chain reaction (PCR, F. Hoffmann la Roche), and in situ PCR.

Alternative amplification-based technologies focus, not on the target nucleic acid itself, but on the amplification of the signal generated in response to the presence of the target. Such methods include, for example, the ligase chain reaction (LCR/Abbott), Q-beta bacteriophage replicase (Genetrak systems), cycling probe technology, (ID Biomedical of Vancouver), b-DNA/ branched DNA (Chiron), in situ hybridization, and ligase hybridization and genomic amplification with transcript sequencing (GAWTS).

All of the above techniques suffer from drawbacks that can render them partially or totally unsatisfactory for incorporation into practical diagnostic systems. Target amplification methods suffer from amplicon and other forms of sample contamination. This can significantly compromise the specificity of the system, and lead to false positive and negative results. Such problems are inherent limitations of their technology bases and consistent with there being few satisfactory kit designs on the market that achieve both high specificity and sensitivity. Additionally, these current technologies cannot efficiently screen for specific RNA targets, which severely restricts the usefulness of these technologies to support recent advances in RNA research in the fields of cancer, and in viral and other infectious disease diagnosis and therapy management.

Furthermore, the upper limit to the amount of total nucleic acid that can be tolerated by most of the assay systems is in the order of just microgram amounts, so that the source nucleic acid harboring a proportionally low amount of the unique target is restricted, and further serves to decrease the specificity and sensitivity of these methods. Indeed, this is of singular importance in diagnosing infectious disease at a stage where antimicrobial agents can be administered to the greatest effect. The earlier in the infectious time course that the detection is desirable, the larger the sample of host DNA (milligram amounts) that is required for analysis, to afford a chance of detecting the rare nucleic acid of the infectious agent.

The signal amplification technologies suffer from similar inherent limitations. Non-specific high background signals and inappropriate levels of sensitivity, due to a total inability to

recognize a target down to a single copy, impede the successful application of these techniques as effective diagnostic tools.

Bioluminescence has for many purposes supplanted radioactivity as the label of choice to mark nucleic acid and currently offers the most sensitive mode of single gene copy detection. Nevertheless, it is only able to detect a minimum of 600,000 copies of the targeted sequences. What is needed is an amplification method of increased sensitivity to the specific target, so that one can detect a single gene molecule, with minimal background interference.

The polymerase chain reaction (PCR) significantly advanced the ability to selectively amplify specific nucleic acid sequences. However, PCR and other amplification techniques are susceptible to sample contamination during the collection procedures, and the presence of amplicons (amplified DNA) which contaminate DNA specimens, lead to false positive results. There are also problems associated with PCR that arise from non-specific target amplification resulting from cross-hybridization of the reaction oligonucleotide primers, specifically intended to recognize the target, and sequences similar to the targeted sequence. The production of primer dimers can also present artifactual and spurious reaction products that reduce the specificity of the procedure. There can be, therefore, reduced control of specificity, creating false positive reactions, and poor control of sensitivity, resulting in false negative reactions. PCR results must often be confirmed and validated by applying additional techniques such as Southern blotting, RNA blotting and probe hybridization, or in situ hybridization, thus complicating the entire assay and introducing other possible error sources. Additionally, PCR amplification reactions can accommodate only very small amounts of starting sample DNA, in the order of 1 microgram or less. This limits the suitability of PCR techniques when the proportion of the target sequence to the total available nucleic acid is miniscule. It is difficult or impossible to detect extremely low copy number nucleic acid targets in a large background of total nonspecific nucleic acids, critical for effective early infectious time-course diagnostics. Optimization of PCR-assay conditions is time-consuming, and selection of the appropriate primers is somewhat arbitrary, and demands extensive experimental confirmation. Minor changes in the protocol readily result in false or misleading data.

Techniques directed towards RNA analysis include Northern blots and the Ribonuclease Protection Assay. For Northern blots, the RNA is denatured and unfolded to a linear form. The RNA is then subjected to gel electrophoresis, transferred to a membrane, hybridized with a labeled probe and subjected to a visualization method. The procedure can be both qualitative and quantitative. However, this lengthy, high cost, procedure has little or no relevance as a practical diagnostic procedure because of those disadvantages it has in common with Southern

blotting, in DNA diagnostics. These include time consumption, high costs of material and labor, lack of sensitivity in detecting targets of relative low abundance, and inability to be sensitive down to a single target copy. In addition, the isolation of RNA of a quality suitable for analysis by Northern blotting is technically demanding and fraught with difficulties of RNA susceptibility to ribonuclease degradation, reducing yield and quality. At best, Northern analysis offers a late infectious time-course assay. At worst, Northern analysis is unsuitable as a routine laboratory diagnostic tool

The Ribonuclease Protection Assay comprises binding a probe to an RNA molecule. S1 nuclease is used to digest single stranded RNA regions not protected by the hybridized probe, and the residual RNA is analyzed on an electrophoretic gel. This procedure, however, is only qualitative and lacks the sensitivity necessary for diagnostic technology due to the amount of RNA needed for effective visualization in the assay.

Reverse transcriptase-PCR (RT-PCR) is the only process currently available with some potential for practical RNA diagnostics, but can achieve only indirect RNA analysis. Typically, the targeted RNA sequence is converted to a DNA copy (cDNA) by a target-specific primer and reverse transcriptase. Quantitative PCR of the target sequence is then performed, but introduces the failings of PCR. RT-PCR has restricted specificity and sensitivity, and requires extreme and fastidious standardization of all steps to obtain reproducible results. As a consequence, it is both labor and cost intensive. Also, RT-PCR cannot be used in early infectious time-course nucleic acid diagnostics.

Thus, methods and compositions are needed that can overcome the current deficiencies in nucleic acid assays that present obstacles to their use as effective diagnostic tools, to detect specific nucleic acid sequences at minimal cost in both time and labor. Especially needed are methods and compositions that provide the flexibility that would allow for isolation of nucleic acid sequences using a desired level of specificity. What is also needed are methods that avoid nucleic acid amplification of the target sequence prior to its detection, but do allow for the isolation of a specific target sequence from any amount of sample nucleic acid, especially large amounts, and have the flexibility to accomplish the isolation at several levels of specificity, depending on the level of specificity desired. What is especially needed are methods and compositions that can detect target sequences using RNA, including, but not limited to, mRNA, as the source of nucleic acid target sequence, and can amplify the signal emanating from the presence of the target nucleic acid, to the exclusion of extraneous and artifactual signals.

Additionally what is needed are signal amplification systems that allow for the detection of specific target nucleic acid sequences and also the detection of other assay end results such as

antibody-antigen interactions. Such signal amplification systems need to be sensitive, accurate and capable of automation.

Summary of the Invention

The present invention comprises compositions and methods directed to analysis and detection of target nucleic acid sequences. Particularly, the methods and compositions comprise formation of duplex and triplex structures. In some embodiments, hairpin structures are employed. These compositions and methods are especially useful in diagnostic assays. The DNA diagnostic embodiments are useful for early detection of infectious disease, cancer, and genetic hereditary testing, while providing high sensitivity and specificity, with time and cost efficiency, and a process design to accommodate ease of automation. Direct RNA diagnostic embodiments are useful for early and sensitive detection of infectious disease, cancer and cancer metastatic states, also providing, high sensitivity and specificity, with time and cost efficiency, and a simplistic process design to accommodate ease of automation.

The present invention also comprises signal amplification systems for nucleic acid target detection and also antigen target amplification. The invention comprises methods and compositions comprises sets for probes that can form amplification structures, MTRF nuclei, and also duplex and triplex reporter probes.

Further, the present invention comprises methods and compositions comprising oligonucleotides including modified nucleotides. These modified nucleotide-containing oligonucleotides can be used in the assays described herein and other related technologies, and are especially useful in triplex formations.

Additionally, the present invention comprises methods and compositions for nonenzymatically resticting nucleic acids using halouracil cutter probes and halouracil hairpin structures. These methods and compositions can be used to restrict nucleic acids at a predetermined site or randomly throughout the genomes.

The methods and compositions of the present invention should be ideal for the detection of viruses and other microorganisms such as pathogens of humans, animals and plants, as well as genetic analysis of polymorphic gene sequences such as HLA typing. The methods of the present invention can be used in forensics, paternity determinations, or transplantation or organs or tissues, or genetic disease analysis. Additionally, the methods and compositions of the present invention can be employed in assays comprising detection of specific binding partners such as antigen-antibody assays. Particularly useful are signal amplification systems for use in ELISA-type immunoassays.

Accordingly, it is an object of the present invention to provide methods to detect specific nucleic acid sequences.

It is yet another object of the present invention to provide methods for detecting specific DNA sequences involving triplex nucleotide structures.

It is another object of the present invention to provide methods for detecting specific RNA sequences involving triplex nucleotide structures.

It is yet another object of the present invention to provide methods for detecting specific DNA sequences involving duplex nucleotide structures.

It is another object of the present invention to provide methods for detecting specific RNA sequences involving duplex nucleotide structures.

It is yet another object of the present invention to provide methods for detecting specific antibody interactions.

It is a further object of the present invention to provide methods and compositions for diagnostic procedures.

Another object of the present invention is to provide a method of detecting nucleic acid sequences involving modified nucleotide-containing nucleic acids.

Another object of the present invention is to provide a method of detecting nucleic acid sequences for the determination of the identity of microorganisms.

It is yet another object of the present invention to provide a method of detecting nucleic acid sequences for the determination of the identity of pathogens.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

Description of the Figures

Figure 1 (I) shows an embodiment of TPA and signal amplification system, wherein the target nucleic acid is mRNA and (II) shows an embodiment of the RFTA and signal amplification system, wherein the target nucleic acid is mRNA.

Figure 2 (I) shows an embodiment of TPA and signal amplification system, wherein the target nucleic acid is DNA and (II) shows an embodiment of the RFTA and signal amplification system, wherein the target nucleic acid is DNA.

Figure 3 shows an embodiment of TPA where a polypyrimidine rich target region of a subspecies specific nucleic acid interacts with a specific double stranded DNA hairpin and its accompanying triplex lock flap, to form a triplex hairpin structure.

Figure 4 shows an embodiment of the Partial Duplex Target Probe (PDTP) complex.

Figure 5 shows an embodiment of TPA wherein the target nucleic acid is mRNA and the labeled triplex reporter provides signal amplification.

Figure 6 shows an embodiment of the Restriction Fragment Target Assay (RFTA), wherein the target nucleic acid is mRNA and the labeled triplex reporter provides signal amplification.

Figure 7A-7B shows an embodiment of TPA wherein the target nucleic acid is mRNA.

Figure 8A-8B shows an embodiment of the RFTA assay system wherein the target nucleic acid is mRNA.

Figure 9A-9B shows an embodiment of TPA where the target nucleic acid is DNA.

Figure 10A-10B shows an embodiment of RFTA where the target nucleic acid is ss DNA.

Figure 11A-11B shows an embodiment of TPA in the reverse dot blot format, wherein the target nucleic acid is DNA.

Figure 12A-12B shows an embodiment of RFTA in the reverse dot blot format, wherein the target nucleic acid is ss DNA.

Figure 13A-13B shows an embodiment of TPA system in the reverse dot blot format, wherein the target nucleic acid is mRNA.

Figure 14A-14B shows an embodiment of RFTA in the reverse dot blot format, wherein the target nucleic acid is mRNA.

Figure 15 shows embodiments of the Multiple Triplex Reporter forming Self-Complexing Probes (MRTF probes); I shows the Poly A region of the mRNA molecule or the Poly dA region of the reporter probe hybridized with Poly dT signal amplification probes to form a reporter triplex; II shows an assembled MTRF

Figure 16 shows signal amplification by the formation of multiple MTRF complexes.

Figure 17 shows an embodiment of an MTRF nucleus.

Figure 18 shows an embodiment of an MTRF complex with multiple nuclei.

Figure 19 shows an embodiment comprising multiple MTRF complexes.

Figure 20 shows an embodiment of MTRF signal amplification in an assay wherein the nucleic acid is DNA.

Figure 21 shows a further embodiment of MTRF signal amplification in an assay wherein the nucleic acid is DNA.

Figure 22 shows another embodiment of MTRF signal amplification in an assay wherein the nucleic acid is DNA.

Figure 23 shows an embodiment of embodiment of MTRF signal amplification in an assay wherein the nucleic acid is RNA.

Figure 24 shows an embodiment of embodiment of MTRF signal amplification in an assay wherein the nucleic acid is mRNA.

Figure 25 shows an embodiment of an MTRF complex that illustrates the sequences of the probes.

Figure 26 shows an embodiment of the Multiple Triplex Reporter forming Self-Complexing Probes (MRTF) where the triplex is stabilized by psoralen cross-links.

Figure 27 shows an embodiment of MTRF directly attached to a specific monoclonal antibody directed to an antigenic target.

Figure 28 shows another view of an embodiment where MTRF is directly attached to an antibody directed to a specific antigen.

Figure 29 is an embodiment of MTRF attached to a secondary antibody which binds to antibodies directed against the target antigen.

Figure 30 shows an embodiment of the BU Cutter Probes that will result in restriction cleavage of the single strand target.

Figure 31 shows an embodiment of the BU Cutter probes that will result in restriction cleavage of the single stranded target.

Figure 32 shows an embodiment of the BU Cutter probes that will result in restriction cleavage of a double stranded target.

Figure 33A-C shows an embodiment of TPA using a modified nucleotide containing "non-hairpin" structure, RP-TFO and MTRF signal amplification.

Detailed Description

The present invention is directed to molecular biological methods and compositions that are used for diagnostic and prognostic determinations. These methods and compositions may comprise nucleic acids that may or may not include nucleotides that are modified chemically, DNA, RNA, proteins, or combinations of nucleic acids that may or may not include nucleotides that are modified chemically, DNA, RNA and proteins.

Embodiments of the present invention are included in the concept of Haystack Processing. Haystack Processing refers to a molecular biological techniques that provide direct analysis of DNA and RNA samples using methods that do not generally use DNA amplification methods, though such methods could be employed with the methods and compositions of the present invention. The sample of nucleic acids potentially containing target nucleic acid sequences is considered a "haystack" in which the target ("the needle") is found. The methods

and compositions of the present invention can be used to analyze up to milligram amounts of nucleic acids. Not only can milligram quantities of mRNA, DNA, or other nucleic acids be analyzed in a single test in the presence of excess of non-specific nucleic acid, but also nanogram, picogram, or femtamole quantities can be assayed for the presence of low copy number targets.

The methods and compositions of the present invention can be used with existing technologies such as PCR (polymerase chain reaction) amplification. The present invention increases the sensitivity of PCR and other amplification-based technologies, as well as nucleic acid chip technology. These technologies can preprocess their samples to be tested, undergo the methods of the present invention to concentrate the targets, preferably in less than one microgram of DNA, and perform their diagnostic test on the target enriched sample using their analysis platforms to yield an even better and higher sensitivity level.

The embodiments of the present invention, including but not limited to TPA, RFTA and CPA, can be used for nucleic acid analysis and differing embodiments can be used in many assay formats, such as gel, tube, dot blot, and others known to those skilled in the art, with high specificity and sensitivity levels.

The present invention comprises detection of specific target nucleic acids. These targets may be detected using any labels and detection systems used for molecular biological techniques. Though specific labeling and detection systems are presented in the examples given, the present invention contemplates the use of any labels or detection systems. Such detection systems include, but are not limited to, enzyme-chromogen reactions, bioluminescence, such as aequorin, renilla and luciferase, chemiluminescence, chemifluorescence, and cold laser based direct dye fluorescence detection.

There are many advantages to the present invention over serology and preexisting nucleic acid analysis technologies. One advantage is high levels of specificity with multiple levels of specificity built into each test, as are shown in Table 1.

Table 1

Platform Assay	Format	Specificity Levels
RNA TPA	Tube	2
	Reverse Dot Blot	3
	Gel	3
RNA RFTA	Tube	3
	Reverse Dot Blot	1
	Reverse Dot Blot (alternate embodiment of above)	3
	Gel	4
DNA TPA	Tube	3
	Reverse Dot Blot	3
	Gel	4
DNA RFTA	Tube	3
	Reverse Dot Blot	3
	Gel	4
PCR	Tube	3
	Gel	3

The present invention also provides methods and compositions for the detection of nucleic acids that comprise a non-amplification based technology. Such a technology has no inherent problems with contamination of negative specimens. Another advantage is that the present invention comprises high levels of sensitivity with the ability to analyze milligram to picogram or smaller quantities of DNA. The present invention also allows for direct nucleic acid analysis (DNA, RNA, mRNA, tRNA and other forms of nucleic acids) that supports analysis of larger nucleic acid samples and does not exclude analysis of very small nucleic acid samples, and target sequences are not lost in the processing, a distinct advantage over indirect analysis.

Various embodiments of the present invention provide other advantages. Immobilized targets are capable of multiple washes, thus assuring fidelity of target processing by preventing interference from nonspecific DNA or RNA, and fidelity of signal amplification, because non-specific signals are prevented. Other advantages include unconnected capture and reporter probes such that the target region joins capture and reporter probes for the analysis, resulting in heightened specificity and sensitivity. The present invention comprises simple processes that have a limited number of steps that aid in reproducibility of results and that are readily automated.

Embodiments of the present invention comprise further embodiments of the Target Protection Assays including Triplex Protection Assays (TPA). Embodiments of TPA were disclosed in U.S. Patent number 5,962,225, which is incorporated herein in its entirety. TPA can be used for direct DNA and RNA analysis by allowing the processing of milligram quantities of nucleic acids to determine low copy number nucleic acid targets. TPA comprises methods and

compositions comprising triplex formation at the target nucleic acid sequence, forming a protected nucleic acid sequence (PNAS). The target sequence is protected from degradation by an enzyme, e.g., EXO III, that will degrade all non-specific DNA, leaving only the triplex target. The methods of TPA can be used in assay formats such as test tube, analytical gel, dot blot, and reverse dot blot formats, and can also be automated.

Other embodiments of the present invention include embodiments of the Restriction Fragment Target Assay (RFTA). Embodiments of RFTA were disclosed in U. S. Provisional Patent Applications, including 60/065,378, 60/075,812, 60/076,872 and PCT Application No. PCT/US98/24226, all of which are herein incorporated in their entireties. The methods and compositions of the RFTA format can be used for the detection of very low copy numbers of nucleic acid targets, either in a vast excess of non-specific nucleic acids or femptamoles of target sequences alone. The invention comprises methods that comprise direct DNA and RNA analysis in the absence of triplex formation for target protection strategy (TPA). This platform technology can be used in assay formats such as test tube, analytical gel, dot blot, and reverse dot blot formats, and can also be automated.

Further embodiments of the present invention comprise the Cutter Probe Assay (CPA) formats. Embodiments of CPA were disclosed in U.S. Provisional Patent Applications 60/065,378, 60/075,812, 60/076,872 and PCT Application No. PCT/US98/24226, each of which is herein incorporated in its entirety. The CPA format can be used for the non-enzymatic restriction of any nucleic acid target. CPA can be used in the detection of very low copy numbers of nucleic acid targets, either in a vast excess of non-specific nucleic acids or in femtamoles of target present alone. The technology platform supports direct DNA and RNA analysis in the absence of triplex formation for target protection (TPA). This platform technology can be used in assay formats such as test tube, analytical gel, dot blot, and reverse dot blot formats, and can also be automated.

One embodiment of the present invention comprises methods and compositions that allow capture and reporter probes to non-enzymatically restrict and excise a unique nucleic acid target. A capture probe is a nucleic acid or oligonucleotide that is capable of immobilizing another nucleic acid. The immobilization can occur through a molecule attached to the capture probe. The molecule is preferably a specific binding partner, such as a biotin molecule, that is capable of interaction with its binding partner, streptavidin. A reporter probe is a nucleic acid or oligonucleotide that either has label molecules directly or that is capable of binding to another nucleic acid to which labeled probe can be bound.

This embodiment comprises methods and compositions using 5 halopyrimidine derivative cutter probes. These modified bases are substituted in the oligonucleotides, particularly in capture and reporter sequences, that hybridize to the target found in the sample or in proximity to the target. Upon activation with UV, x-ray or gamma radiation, the substituted bases form two free radicals. The Uracilyl free radial cleaves the phosphate-sugar backbone of the probe (capture and reporter) and, the second radical, a Bromine free radical, breaks the opposite strand sugar-phosphate backbone. This cleavage results in the restricting of the target nucleic acid and upon complete restriction of both strands, target excision occurs. This effect may be heightened by the simultaneous addition of the cutter probe and modified halouracil polyamide hairpins to the minor groove juxtaposed to the BU position in the cutter probe upon forming a duplex or a triplex with the target nucleic acid to be restricted.

Another approach to restriction of nucleic acids in vivo and in vitro is to hybridize a duplex nucleic acid target with a triplex forming oligonucleotide (TFO) containing a modified halouracil base (herein referred to as BUTFO) which, upon radiation and free radical formation, causes the double strand scission of the nucleic acid.

Single and double strand nucleic acid non-enzymatic restriction can be supported by a synergistic effect by use of a minor groove binding modified halouracil substituted or BU (bromouracil)-linked to other bases, and this substitute polyamide amino acid hairpin can be used in concert with the BU cutter probes and the BUTFOs to generate multiple sources and numbers of free radicals in strategic base stacking planes.

Use of the modified halouracil substituted polyamide hairpins assists cleavage of any nucleic acid structure possessing a minor groove, such as a single strand nucleic acid forming a duplex with a 5BU (5-bromouracil) cutter probe or a double stranded nucleic acid forming a triplex with a BUTFO in vitro. Furthermore, use of these substituted hairpins could assist in vivo nucleic acid restriction by their entry into the cell without degradation by membrane bound cellular nuclease, similar to the use of sulfur-backbone TFOs. These modified bases delivered by either a hairpin or TFO participate in targeted cell death.

Though not wishing to be bound by any particular theory, a method of activity of such in vivo restriction occurs by the following theory. The polyamide hairpins of MW < 1200 penetrate the cell and recognize a 7 base specific DNA sequence. A region of >7 bases is targeted by addition of 2 or more hairpins that function in concert to destroy the entire target region. Furthermore, it has been demonstrated that the specificity or stability of the hairpin minor groove binder does not increase with sizes greater than that will be accommodated by the 7 base pair minor groove region.

The BUCP (Bromouracil cutter probe) can be delivered by a substituted DNA or RNA molecule for restriction of ss (single-stranded) nucleic acid and BUTFO cleavage of a nucleic acid duplex, and can also be delivered by any DNA binding synthetic ligand that can specifically discriminate the 4 bases, such as the case in the polyamine hairpins.

Another approach would be to use the minor groove binding polyamine hairpins to carry a 5 halopyrimidine derivative that itself would aid in the restriction of the target (w) strand by binding in the minor groove, specifically at the ends of the target sequence to be restricted and these would exist on opposite ends of the capture and reporter probes. Upon UV or x-ray or irradiation of the halopyrimidine derivative, free radicals would be formed that may in cleave the in vivo or in vitro DNA duplex.

In some situations, the BU polyamide hairpin, which binds to the minor groove, provides optimal restriction of ds nucleic acids. In other situations, the BUTFO, which binds in the major groove, provides optimal restriction of ds nucleic acids. When both the BU polyamide hairpin and the BUTFO are used, there is a synergistic effect for cutting of ds nucleic acids.

Use of the 5Bu substituted polyamine hairpins is herein described. 5Bu, is the most preferable halopyrimidine derivative selected for hugh frequency double strand break generation in a DNA triplex or duplex, or any other structure possessing major and minor groove structures. Such cleavage in vitro, can be used for in vivo restriction and even participate in targeted cell death, similar to the use of sulfur-backbone TFOs to allow entry into the cell without degradation by membrane bound cellular nuclease.

The modified bromouracil or linked bromouracil base in BUCP and BUTFO can not only be delivered by a substituted DNA or RNA molecule allowing restriction of ss nucleic acid and BuTFO cleavage of a nucleic acid duplex, but can also be delivered by any DNA binding synthetic ligand that can specifically discriminate the 4 bases, such as the case in the polyamide hairpins or peptide nucleic acids. An embodiment of RFTA with the target restriction achieved non-enzymatically by a DNA bound cutter free radial producing halouracil, is CPA.

Herein, DNA TPA, DNA RFTA, mRNA TPA, and mRNA RFTA will be presented in the test tube, analytical gel, and reverse dot blot embodiment formats with special attention to the novel triplex reporter method for signal amplification, i.e., forming the reporter triplex Poly dT • Poly dA • Poly dT or Poly dT • Poly A • Poly dT.

All diagnostic assay formats have their overall advantages and disadvantages. Test tube assay formats offer high sensitivity, but are often cumbersome if testing a large number of specimens and multiplexing for a number of different diagnostic targets. Gel assay formats offer less sensitivity and similarly are cumbersome for testing of a large number of specimens.

Another assay format called Reverse Dot Blot can provide increased sensitivity over the gel format while supporting rapid testing of multiple specimens with the capability of multiplexing for identification of multiple targets in a single specimen. Selection of an appropriate assay format is well within the knowledge and skill of those skilled in this art and the present invention is not limited to any one particular assay format or only to those described herein. The methods and compositions of the present invention can be used with many different assay formats known to those skilled in the art.

It is important to understand that the methods and compositions of the present invention can be customized and designed to accommodate the anticipated target numbers in the sample and the sample size to be tested and deliver a combination of specificity, sensitivity, and speed to achieve diagnostic success.

Following are embodiments of TPA and RFTA processes. DNA and mRNA formats are described for both processes with special attention to signal amplification for target detection. Each process format will be presented with designated levels of specificity. A particular definition of specificity is offered herein, though the present invention is not to be limited by such definition. The level of specificity is defined and is used to compare TPA and RFTA technology to preexisting DNA analysis technology (PCR).

The level of specificity can be defined as a unique and ordered event that must occur in order to visualize the test result and is a direct result of the presence of the target. In diagnostic testing, the result would be highly specific detection of the DNA target.

A comparison of the TPA and RFTA assays are shown in Figures 1 and 2. Figures 1-I and 1-II illustrate the TPA and RFTA mRNA formats respectively. Figures 2-I and 2-II illustrate TPA and RFTA DNA formats respectively.

The target/probe detection complexes for mRNA TPA and mRNA RFTA for test tube and gel formats can be seen in Figure 1. The numerical designations represented in Figure 1-I represents the PNAS structure of the mRNA TPA process, as follows:

1, the mRNA specific subspecies molecules containing the target within the 5' end coding region.

2, the duplex ds DNA hairpin with the triplex lock

3, the remainder of the encoding 5' end of the mRNA

4, the non-specific mRNA region between the 3' poly A end and the 5' end coding region target inclusive.

5, the polypyrimidine target, part of the 5' coding region of the mRNA molecule.

6, the PNAS/tails structure or the target/probe complex in the TPA method for detection of the target nucleic acid.

7, Biotin is the biochemical hook used to attach the biotinylated capture probe bound target to a solid support.

8, the DNA/RNA heteroduplex of the triplex lock flap.

9, the restriction site in the hairpin described herein.

10, the Poly A (~250 mer) for the triplex reporter formation.

11, the Poly dT signal amplification probes (pair) 40 mer and conjugated with a label.

12, Poly dT (40 mer) 5'-3' hybridizing to the Poly A region to form the RNA/DNA heteroduplex (highly stable structure) attaching additional labels to the PNAS.

13, Poly dT (40 mer) 3'-5' hybridizing to the Poly A • Poly dT duplex region to form the stable reporter triplex structure attaching additional labels to the PNAS.

14, the reporter triplex structure poly dT (3'-5') • Poly A (3'-5') • Poly dT (5'-3') with a minor groove available for polyamide hairpin binding

15, Label, can be DIG which is a binding pair partner attached to the poly dT signal amplification probes in this figure and can be attached to the polyamine hairpins (minor groove binders) to assist signal development and complex detection.

16, Means for attaching the capture molecule, such as a covalent bond or linker.

Numerical designations represented in Figure 1-II represent the PDTP (Partial Duplex Target Probe) structure of the mRNA RFTA process, follow:

1, the mRNA specific subspecies molecules containing the target within the 5' end coding region.

2, the target varying in 4 bases and found in the 5' end mRNA coding region.

3, the non-specific mRNA region between the 3' Poly A end and the 5' end coding region, target inclusive.

4, The Poly A region.

5, The primary probe composed of two regions, one, a 5' end complementary to a target of variable size ,18 bases and, the second, 8, a 3' end that is Poly dA ~120 mer. Alternatively, the probe has a 3' end that is capped with a 5' poly A end to render this probe nuclease resistant.

6, the secondary probe in this embodiment is a biotinylated capture probe complementary to any part of the 5' encoding region of the mRNA, not including the region binding the primary probe.

7, Biotin is the biochemical hook conjugated to a capture probe and used to attach the PDTP complex to a solid support.

8, Poly dA (~120mer), in this embodiment, is the region of the reporter probe that will form the structural core for the triplex reporter.

9, the Hoogsteen Bonding used to stabilize the interaction between the Poly dT (3'-5') and the heteroduplex Poly A (3' to 5') • Poly dT (5'-3')

10, the Poly dT is the signal amplification probe used to create the reporter triplex with numerous labels conjugated to it.

11, the signal amplification probe Poly dT (40 mer) 3'-5' forming a duplex with the Poly dA (5'-3')

12, the signal amplification probe poly dT (40 mer) 5'-3' forming a triplex by interaction with the duplex, Poly dA (5'-3') • Poly dT (3'-5')

13, Label, which can be DIG a molecule attached to the Poly dT probes, the polyamine hairpins (minor groove binders), and anti DIG is the complementary affinity molecule used as a signal producing conjugate to assist signal development and complex detection.

14, the Partial Duplex Target/Probe Complex (PDTP complex) of RFTA, the target/probe detection complex for RFTA test tube and the gel formats.

15, the reporter triplex Poly dT(5'-3') • Poly dA (5'-3') • Poly dT (3'-5') with a minor groove available for the polyamide hairpin binding.

16, Means for attaching the capture molecule, such as a covalent bond or linker.

17, 5' cap on mRNA

The target/probe detection complexes for DNA TPA and DNA RFTA for test tube and gel formats can be seen in Figure 2. Numerical designations represented in Figure 2-I represent the PNAS structure of the DNA TPA process, follow:

1, the duplex target DNA

2, a Triplex Forming Oligonucleotide (TFO) with sequence specificity for the target duplex

3, The Protected Nucleic Acid Sequence (PNAS) formed by the hybridization of the target with the TFO

4, the capture region is either of the 5' tails attached to the PNAS/tails structure and the capture probe is complementary to this sequence.

5, the reporter region is the other 5' tail attached to the PNAS/tails structure and the reporter probe has a section complementary to this sequence.

6, the capture probe is conjugated with the biotin molecule to facilitate attachment to a streptavidin coated solid support.

7, Biotin is the biochemical hook which is conjugated to the capture probe.

8, the reporter probe has two sections, one, the sequence complementary to the reporter region and, 9, a 3' poly dA region (~120 mer) or in other embodiments, which can have a 3' capped or 5' capped end, rendering the reporter probe nuclease resistant.

9, the poly dA sequence which forms the structural support for the reporter triplex (poly dA 5'-3')

10, the Poly dT signal amplification probes (pair) conjugated with an affinity molecule (ex. DIG)

11, the signal amplification probe conjugated Poly dT (3'-5') 40 mer that will form the duplex Poly dA (5'-3') • Poly dT (3'-5'), antiparallel orientation.

12, the signal amplification probe Poly dT (5'-3') 40 mer that will form the reporter triplex with the duplex Poly dT (5'-3') • Poly dA (5'-3') • Poly dT (3'-5'), parallel orientation.

13, Label, which can be DIG is a molecule conjugated to the Poly dT probe pair and the polyamide hairpin structures to provide attachment of additional label molecules through use of an anti DIG - label conjugate.

14, the reporter triplex, Poly dT • Poly dA • Poly dT with a minor groove available for polyamide hairpin binding.

15, the PNAS/tail structure

Numerical designations presented in Figure 2-II represent the PDTP complex of the DNA RFTA process, follow:

1, the DNA target sequence (the watson, w, strand)

2, the primary probe has 2 sections, one that hybridizes to a region at the target 3' end and, **4,** that has a sequence complementary to the reporter probe.

3, the capture region has a sequence complementary to the biotinylated capture probe

4, the reporter region has a sequence complementary to the reporter probe.

5, the capture probe complementary to the capture region (5' end of target) and which has a molecule (biochemical hook) for binding of the probe

6, A molecule for immobilizing the structure via the capture probe, e.g. biotin is the biochemical hook.

7, the reporter probe has two sections, one, the 5' end has a sequence complementary to the reporter region, and, 9, the 3' end has a poly dA sequence (~120 mer) which forms the structural core for the reporter triplex formation.

8, normal hydrogen bonding is the only bonding present in the PDTP complex; however, if a reporter triplex is generated, by the poly dT advanced reporter system then Hoogsteen Bonding would be also found attaching the TFO, poly dT, to the purine (w) strand of the Poly dA region.

9, the Poly dA region supporting signal amplification by generation of the reporter triplex.

10, the Poly dT signal amplification probes (pair) 40 mer

11, the Poly dT (3'-5') 40 mer signal amplification probe conjugated with DIG (a label) and forming the duplex Poly dA • Poly dT, antiparallel orientation.

12, the Poly dT (5'-3') 40 mer signal amplification probe conjugated with DIG (a label) and forming a triplex reporter Poly dT (5'-3') • Poly dA (5'-3') • Poly dT (3'-5'), parallel orientation.

13, Label, e.g., DIG is the molecule used, conjugated to the Poly dT probe pair and the polyamide hairpin structures to attach label molecules to the PNAS via a label-anti DIG conjugate.

14, the reporter triplex Poly dT • Poly dA • Poly dT with a minor groove available for polyamide hairpin binding.

15, the PNAS (protected nucleic acid structure)

Sizes of DNA or RNA probes or targets are variable with the embodiments presented herein being representative of these assays based on the technology being discussed. In no manner is it intended that the lengths of all probes, all targets, and all regions be limited. The lengths of particular probes or sequences can easily be determined by those skilled in the art.

Direct Detection of a mRNA Unique Subspecies

Direct detection of low copy number mRNA targets has been unattainable by current DNA analysis technologies. The best technology available, prior to the present invention, is called Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), and has many drawbacks.

Both TPA and RFTA methods and compositions can be used to directly analyze mRNA populations in general for a specific and unique sub-population comprising the target. In these processes direct mRNA analysis relies on the presence of a unique region on a specific mRNA sub-population of molecules or targets.

In the TPA methods, preferably, a region of about 18 nucleotides, that is polypyrimidine rich, is targeted for triplex formation by interaction with a specific double stranded DNA hairpin and its accompanying triplex lock flap. The target region of the subspecies specific mRNA molecule and the triplex hairpin structure are presented in Figure 3. The numbered structures represent the following:

1, the polypyrimidine mRNA subspecies specific target

2, the mRNA molecule-subspecies specific

3, the polypurine (w) strand of the duplex hairpin.

4, the polypyrimidine (c) strand of the duplex hairpin

5, the hairpin loop region, usually five thymine molecules (single stranded)

6, the normal hydrogen bonding found between the w and c strands of the ds DNA hairpin and between the mRNA (non-target region) and the triplex lock flap with inverted polarity.

7, the Hoogsteen Bonding between the polypyrimidine target and the polypurine Watson (w) strand.

8, the endonuclease restriction site whose 5' end is at least partially included within the polypyrimidine mRNA target region.

9, DNA bases with normal polarity

10, DNA bases with reverse polarity that comprise the triplex lock flap that form a duplex with an upstream region (toward the 3' end) of the mRNA that is in proximity to the target (usually adjacent).

11, any structure (or bonding method) to connect two oligonucleotides of opposite polarity - a linker molecule.

12, the PNAS (the polyrich region of the hairpin hybridized to the polypyrimidine mRNA target sequence.

13, normal DNA varying in 4 bases forming duplexes (with normal polarity /antiparallel) between the DNA hairpin and the subspecies specific mRNA.

14, the restriction endonuclease cleavage site (in this case, APA 1)

15, change in polarity site

16, the encoding 5' end of the mRNA molecule

17, the non-encoding 3' poly A end of the mRNA molecule.

18, non-species specific mRNA region

In the RFTA methods, a region conferring specificity is selected. A preferred sequence would comprise 18 nucleotides. It may vary in 4 bases (A,T,G,C) or it may vary in 2 bases(A,G)

or (T,C) being designated polypurine or polypyrimidine rich. A major advantage to RFTA methods and compositions is the ability to recognize the 4 base variation regions opposed to the 2 base varying polyrich regions. Furthermore, the Partial Duplex Target Probe complex (PDTP complex) has less stability concerns than the PNAS of TPA. A preferred embodiment of the PDTP complex is depicted in Figure 4. The numerical designations represent the following:

1, the subspecies specific mRNA (carrying the target sequence).

2, the target varying in 4 bases (normal DNA) or 2 bases (polyrich region)

3, the primary probe which in this embodiment is composed of a 5'or a 3' capped poly dA end of ~120 mer and a 3' end complimentary to a segment of the target.

4, the secondary probe which in this embodiment binds antiparallel to the 5' mRNA end (coding region only) and has a molecule of biotin attached covalently as a biochemical hook to function as the capture molecule to capture the structure

5, the biochemical hook or capture molecule, e.g., biotin

6, the Poly dA ~120 mer region to be used as a core for amplification of the target signal to facilitate detection of the presence of the target.

7, normal hydrogen bonding between the primary and secondary probes and their attachment to the targeted mRNA subspecies specific population.

8, the Poly A (~250 mer) RNA region located on all mRNA molecules (3' end) that will be degraded by exoribonuclease (single strand mRNA specific 3'- 5' degradation) up to the 3' end of the selected target region that is a duplex with secondary structure.

9, the non-specific mRNA region varying in 4 bases that is found between the 3' Poly A region and the 5' encoding end subspecies specific region.

10, Means for attaching the capture molecule, such as a covalent bond or linker.

The Cutter Probe Assay is an embodiment of RFTA where the capture and reporter probes also function to restrict the target non-enzymatically by the use of a free radical forming derivative of 5 Bromouracil (5Bu). Again mRNA CPA direct analysis and gel and tube formats have been provided. DNA CPA was also described for gel and tube formats.

The examples given herein refer to mRNA. It is to be understood that any RNA can be used in such examples. Areas where poly A regions are used in the examples in mRNA, poly A regions can either be added to the other RNAs or other suitable modifications to the RNAs that function like the poly A regions can be made. Additionally, capping of ends can be replaced by methods that prevent nuclease degradation.

Another embodiment of the mRNA-TPA application involves the use of a duplex double hairpin with the 3' end juxtaposed to the 5' end in the middle of the duplex. Since EXO III can function at nicks in double stranded DNA to produce single strands, then hairpins that do not bind mRNA target TFOs are readily degraded. Conversely, binding of the mRNA target TFO to the duplex double hairpin will preclude the activity of EXO III by covering the nick (joined 3' and 5' ends) thus rendering the triplex (mRNA and double hairpin duplex) EXO III resistant.

Direct detection of low copy number mRNA targets is also dependent upon amplification of the signal used to detect presence.

Signal Amplification: The Duplex Reporter, the Triplex Reporter, Hairpins and MTRF Complexes

In TPA and RFTA, a similar signal amplification approach has been developed and is represented in Figure 5 (TPA signal amplification) and Figure 6 (RFTA signal amplification). The specific label for signal detection can be any label known to those skilled in the art. Presented, herein, is a signal amplification triplex reporter that is readily formed with minimal detectable background and an ability to recognize mRNA targets down to very low copy number.

When the label is an enzyme, there is a definite signal amplification advantage. The known enzyme turnover rates of substrate molecules can easily reach thousands per minute per enzyme molecule. Having a number of enzyme molecules stably attached to the target would allow the accumulation of many logs of modified substrate molecules, which could be detected by chemiluminescence, chemifluorescence, soluble or insoluble, chromogen formation, and other techniques known to those skilled in the art. Use of an enzyme label may increase the non-specific background signal. In the examples shown, DIG is used as the label, and any label is contemplated by the invention, not just the DIG label shown.

The first step in signal amplification by use of Poly dT (40 mer) probes is the formation of the duplex:

for mRNA-(antiparallel binding)

Poly A (3'-5') • Poly dT (5'-3')

for DNA-(antiparallel binding)

Poly dA (3'-5') • Poly dT (5'-3')

This allows additional labels to be attached to the PNAS and PDTP complexes.

The second step in signal amplification by use of Poly dT (40 mer) probes is the formation of the triplex:

for mRNA-(parallel binding)

Poly dT (3'-5') • Poly A (3'-5') • Poly dT (5'-3')

for DNA-(parallel binding)

Poly dT (3'-5') • Poly dA (3'-5') • Poly dT (5'-3'

This allows double the number of multiple labels to be attached to the PNAS and PDTP complexes.

Numerical designations presented in mRNA TPA in Figure 5 are as follows:

1, the mRNA subspecies specific population

2, the polypyrimidine mRNA target sequence

3, the 5' encoding region of the mRNA molecule

4, the PNAS (protected nucleic acid sequence)

5, the non-specific mRNA region

6, the ds DNA hairpin with the triplex lock flap

7, the triplex lock flap

8. the Poly A region (~250 mer) located on the 3' end of most mRNAs.

9, the Poly dT signal amplification probe (5'- 3') 40 mer structure conjugated with Digoxygenin (DIG) at least at every fifth base (8 DIG per probe) forming a duplex by binding anti-parallel to the poly A region with normal hydrogen bonding (Poly A • Poly dT).

10, the Poly dT signal amplification probe (3'–5') 40 mer structure conjugated with Digoxygenin (DIG) at least at every fifth base (8 DIG per probe) that binds parallel to the Poly A (mRNA) region with Hoogsteens bonding forming a triplex. (Poly dT • Poly A • Poly dT).

11, Label, e.g.,Digoxygenin (DIG) used as a molecule to bind the antibody-enzyme conjugate for signal detection.

12, the endonuclease restriction site partially located in the polypyrimidine target.

13, the biochemical hook, biotin

14, the streptavidin protein attached to a solid support for attaching targets (biotin/streptavidin interaction)

15, the solid support

16, the poly dT secondary signal amplification probes

Strategies for signal amplification in RFTA assays similarly involve triplex reporter formation.

Numerical designations in the PDTP complex schematic presented in Figure 6 are as follows:

1, the mRNA subspecies specific population

2, the pyrimidine target region

3, the 5' encoding region of the mRNA

4, the primary probe containing a ~120 mer Poly dA region, and a 3'capped or 5' end, that forms the PDTP complex

5, Hoogsteen Bonding between the Poly dT (5'- 3') and the (5'- 3') Poly A regions (binding parallel) resulting in triplex formation

6, Normal Hydrogen Bonding between the Poly dT (3'- 5') and the (5'- 3') Poly A complex (antiparallel) resulting in duplex formation.

7, the biochemical hook, biotin

8, the Poly dA region (~120 mer) used for signal amplification in this embodiment.

9, the Poly dT signal amplification probe (5'-3') 40 mer structure conjugated with Digoxygenin (DIG) at least at every fifth base (8 DIG per probe) and binds parallel to the (5'- 3') Poly dA via Hoogsteen's bonding to the duplex Poly dA • Poly dT, forming the triplex reporter.

10, the Poly dT signal amplification probe (3'- 5') 40 mer structure conjugated with DIG at least at every fifth base (8 DIG per probe) and binds antiparallel to the (5'- 3') poly dA via normal hydrogen bonding forming a duplex reporter.

11, Label, e.g., the Digoxygenin (DIG) used as affinity molecule to bind the antibody-enzyme conjugate for signal detection.

12, the Poly dT secondary signal amplification probes

13, the triplex reporter

14, mRNA 3' end which is a substrate for 3'→5' ss exonuclease

15, protected end of probe, unable to be degraded by nucleases

To reiterate, the signal amplification strategies for TPA and RFTA result from a multi-step process. The first involves the antiparallel binding of the Poly dT (3'-5') probes (40 mer) resulting in stable duplex formation (Poly A or Poly dA • Poly dT). The second step involves the parallel binding of the Poly dT (5'-3') probes (40 mer), to the Poly A or Poly dA strand of the duplex, resulting in stable triplex formation. A third step can involve the binding of label substituted polyamide hairpin molecules to the minor groove of the reporter triplex.

Binding of Polyamide Hairpins Conjugated with the Label to the Minor Groove of the Reporter Duplex

Polyamides containing N-methylpyrolle and N-methylimidazole amino acids are synthetic DNA binding ligands that have the same affinity and specificity for DNA as other naturally occurring DNA binding proteins (enzymes, histones, etc.) These ligands are known to

associate with DNA in a 2:1 ratio in a stable manner when configured as a hairpin polyamide that folds back over on itself and bind to the floor of the minor groove of the DNA double helix.

It has been demonstrated that the amino acid pairs (Py-aromatic Pyrolle, Im = Imidazole) recognize the following bases: Im/Py specifically recognizes G-C pairs; Py/IM specifically recognizes C-G pairs; Py/Py is degenerate in that it cannot discriminate the difference between A•T or T•A pairs. The degeneracy has been corrected by a modification in the hairpin connection region. From the standpoint of current direction, interest lies in the ability of the polyamide hairpin molecules to bind to the minor groove floor in the reporter triplex Poly dT•Poly A or Poly dA•Poly dT.

The 3' mRNA or other Poly A region can be hybridized by Poly dT conjugated with at least one Digoxygenin (DIG) every fifth base. Though digoxygenin is used herein and elsewhere in the disclosure, it is only for illustration, other lablels or molecules can function in the same manner are contemplated in the present invention. Formation of the Poly A•Poly dT heteroduplex would deliver up to six poly dT (40 mer) probes carrying at least 8 DIG molecules, a total of 48 DIG secured to the target heteroduplex formation. Furthermore, by reporter triplex formation (hybridization of the Poly dT (40 mer) TFO with the heteroduplex, Poly A• Poly dT) a similar effect of delivery of up to an additional 48 DIG molecules are bound to the target nucleic acid.

Another embodiment of the reporter triplex comprises polyamide hairpins that can deliver additional label molecules to the reporter triplex to aid in signal amplification. The rationale is as follows: one, the hairpin (polyamide) that is designed to recognize a five base sequence would follow the rules known by those skilled in the art for specificity determination. An example of a polyamide hairpin that would recognize a Poly A • Poly dT or Poly dA •Poly dT duplex would be represented by the following design:

PyPyPy-V-(R)H2N Py-PyPyPy-B-Dp

which binds to the floor in the Poly A or dA• Poly dT minor groove, where Py=Pyrolle, V-(R)H2N=V- derivative of aminobutyric acid, hairpin connection region, B=beta-alanine, Dp=Unique hairpin end structure.

The maximum number of DIG molecules that can be conjugated to the hairpin can be determined and for this embodiment a single DIG is conjugated to each hairpin in any position known to those skilled in the art. This embodiment would allow the binding of up to 48 polyamide hairpin molecules to a 240 nucleotide Poly A reporter triplex delivering up to an additional 48 DIG molecules.

In this embodiment of the reporter triplex, the individual contributors to the total number of DIG bound to the target/probe complex (TPA's, PNAS and RFTA's PDTP Complex are as seen in Table 2:

Table 2

Poly dT Binding	DIG Bound Every 5 Bases	Total DIG per dT	Total DIG from Duplex Formation
	6	6.6	39.6
	5	8	48
	4	10	60
	3	13.3	79.8
Poly dT Binding	DIG Bound Every 5 Bases	Total DIG per dT	Total DIG from Triplex Formation
	6	6.6	39.6
	5	8	48
	4	10	60
	3	13.3	79.8
6 Ring Polyamide Hairpin Binding	DIG Bound per Hairpin	Total DIG per Size Heteroduplex	Total DIG from Polyamide Hairpin Binding
	1	48	48
	2	96	96

Signal Amplification and the Components of the Reporter Triplex.

In addition, if even more signal amplification is needed, the present invention contemplates the use of DNA intercalating dyes such as ethidium bromide. This dye, upon UV irradiation, produces a characteristic fluorescence in solution; while bound in the stacking basis of the helix/duplex the interacting bases of the triplex, and the crevices in the minor groove surrounding the bound polyamide hairpins. The fluorescence intensity increases and can assist signal amplification for the concentration of dye molecules in the target structure is greater than that in the solution background. Such use of these dyes is compatible with the capture and wash steps in TPA, RFTA, and CPA.

The overall signal amplification generated could be a function of, one, the direct attachment of fluorescent molecules to the DIG labels on the reporter triplex and, two, the additional fluorescence produced by the intercalating dye molecules. Theoretically, the DIG molecules in the duplex and triplex stages as well as the polyamide hairpin minor groove binding could deliver a minimum (based on DIG conjugated to every 6 bases) of 79.2 DIG and a maximum (based on DIG conjugated to every 3 bases) of 239.4 DIG (approximately 2.5 logs), see Table 2.

The intercalating dye fluorescence should be on the additional order of magnitude of one to two logs. Signal amplification can be delivered in the 5-6 log range, which would provide sufficient signal amplification to detect very low copy numbers of nucleic acid targets. This could be achieved using bioluminescence, chemiluminescence , chemifluorescence, direct fluorescence (dye and other) and direct cold laser detection of dyes emitting in the fluorescence region.

The current status of Aequorin bioluminescence requires over 600,000 enzyme molecules connected to a target to identify its presence, requiring one molecule of enzyme per 600,000 targets. Subsequent binding of the anti-DIG-Aequorin conjugate and the target/probe/protein complex will attach numerous enzyme molecules to the target. Use of the reporter triplex can provide up to a 5-6 log signal amplification; thus, use of the anti-DIG-Aequorin conjugate allows detection of as few as 3 to 30 targets never before achievable. The importance in diagnostics is the absolute specificity and sensitivity of the assays. An element effecting the use of the reporter triplex is the stability of the triplex complex. Since the triplex forming oligonucleotide (TFO) lies in the DNA duplex major groove and is held in place by Hoogsteen's Bonding (weak hydrogen bonds), stabilization of the triplex formation in vivo and in vitro can be used with methods known to those skilled in the art.

The Poly dT • Poly dA • Poly dT triplex has been shown to possess a deep major groove in the duplex form, and binding the TFO to the major groove almost completely encloses it in the groove itself. This supports good triplex stability; however, tactics such as halopyrimidines substitution in the TFO have been shown to increase triplex stability at physiological pH by adding to the charges on the DNA duplex and furthering the formation of its hydrophobic spine. Also, triplexes containing Poly BU (5BU) • Poly dA • Poly dT evidence increased stability over Poly dT • Poly dA • Poly dT as well as triplexes containing 5mC in the pyrimidine TFO, and even the use of photopsoralens to crosslink the reporter triplexes to increase stability. Furthermore 5-aminoadenosine and 5-aminoguanosine when substituted into a polypurine region of a triplex will better support the stabilization of the triplex at physiological pH (discussed elsewhere in this document).

Furthermore, selection of the T•A•T triplex does not preclude use of any other polynucleotide triplex format including those containing Adenine, Thymine, Guanine, Cytosine and Inosine (I).

The Poly dC • Poly dG • Poly dC triplex can be used with 5 methyl cytosine in place of cytosine in the TFO third strand. Therefore, the triplex would be Poly dmC • Poly dG • Poly dC, the increased stability is also due to the additional charges the methyl groups contribute to

the triplex helical structure which will help create a tightened hydrophobic spine in the triplex further enhancing stability.

The signal amplification scheme presented herein can be configured to work with the variety of detection systems currently in use. Such systems include bioluminescence (Aequorin and others). chemiluminescence, chemifluorescence, detection of heavy metal chelates and direct fluorescence detection by cold laser.

Use of the signal amplification triplex in TPA and RFTA can readily attach many label molecules to the target nucleic acid, which can immediately improve target detection down to hundreds of copies using aequorin bioluminescence and with other more sensitive detection technologies (cold laser and dye) down to single gene copy and single mRNA copy.

mRNA TPA: Test Tube Format

Figure 7A and 7B represents mRNA TPA, test tube format. This process includes the following steps:

Step I: Isolation of mRNA by any method known by those skilled in the art. A polypyrimidine target site could also be selected. See Fig. 7A 1)

Step II: Hybridization of a TFO (ds DNA Hairpin) with the triplex lock. The combination of the target sequence and the ds DNA hairpin form the PNAS (protected nucleic acid sequence). See Fig. 7A 2)

The triplex lock hairpin duplex probe has no available 3' ends that would allow Exonuclease III to destroy the protected target. To achieve this, the previous 3' end of the hairpin duplex has been converted to a 5' end by a change of polarity across a spacer molecule, thus converting the 3' hairpin end to a 5' end. This is accomplished by any method that can join two oligonucleotides of opposite polarity. In DNA TPA, the probe protects the target and in mRNA TPA, the target protects the duplex probe from degradation.

Step III: Nuclease destruction of unbound ds DNA hairpin probes. Subspecies specific mRNA/Hairpins complex (PNAS) survives treatment with the exonuclease. Hairpins not protected by the target are cut at the restriction endonuclease site, followed by degradation by EXO III completely destroying all nonspecific hairpins. Both enzyme treatments can be considered together as a single level of specificity. (First level of Specificity) See Fig. 7A 3)

Step IV: EXO III treatment to degrade the, unprotected, restricted hairpin (newly exposed 3' ends). Both the restriction endonuclease and EXO III treatments together may be considered a level of specificity. (Second level of specificity) See Fig. 7B 4)

Step V: Anchor the PNAS to a solid support through the biotin-streptavidin interaction or any other such specific binding partner pair. See Fig 7B 5)

Step VI: Wash to remove non-target mRNA enzyme and restricted probe.

Step VII: Hybridize the Poly A region with the Poly dT signal amplification pairs, conjugated with DIG at least every 5 bases under conditions to form the triplex Poly PY • Poly PU • Poly PY. See Fig. 7B 6)

Up to 96 label molecules can be attached to the mRNA (3') Poly A end by triplex formation with labeled poly dT (5'-3') and Poly dT (3'-5'). A Poly A (~250 mer) segment may bind up to 12 poly dT (40 mer) molecules (3'-5') and (5'-3') each carrying at least 8 DIG labels (every fifth base). Therefore, the reporter triplex is composed of the Poly dT (5'-3') (6 x 40 mer (nucleotide) units) • Poly A (one 250 mer unit) • Poly dT (3'-5') (6 x 40 mer units) resulting in up to 96 labels attached to the target.

Whatever the signal amplification technology used, the signal background should be kept at a minimum.

Step VIII: Wash to remove unbound probe.

Step IX: Add the IgG anti-DIG/enzyme conjugate and use any signal recognition technology.

Step X: Wash to remove excess unbound conjugate

Step XI: Add a chromogenic substrate, which upon enzyme interaction produces a soluble product. Quantitate by measurement of absorbency or by direct fluoresence or other detection methods.

The numerical designations for mRNA TPA Test Tube Format in Figures 7A to 7B are as follows:

1, mRNA subspecies specific polypyrimidine target

2, 5' encoding end of mRNA molecule (usually capped/confers nuclease resistance to the 5' end).

3, non-target specific mRNA region (non-target sequences)

4, Poly A ~250 mer located on 3' end of some RNA molecules, specifically mRNA

5, the 5' mRNA capped end that is also nuclease resistant

6, 5' mRNA nuclease resistance (capped end)

7, Triplex lock (ds DNA Hairpin)

8, Polypyrimidine (c) strand of the ds DNA Hairpin duplex

9, Two base variation in the PNAS region

10, Polypurine (w) strand of the ds DNA duplex hairpin

11, Triplex lock/region of reverse polarity on the DNA probe that will bind to an upstream sequence of mRNA by formation of a stable hydrogen bonded DNA/RNA heteroduplex.

12, mRNA Triplex lock at least 18 bases with variation of all 4 bases (A,T,G,C)

13, 5' (w) strand end - the hairpin possesses no 3' ends conferring resistance to EXO III degradation (no 3' end available) and is generated by a polarity reversal.

14, the restriction site (the 5' end should be partly or wholly buried in the polyrich region of the ds DNA hairpin.

15, the PNAS (protected nucleic acid sequence)

16, the biochemical hook – biotin

17, DNA/RNA Heteroduplex formation is the mechanism of triplex lock mechanism and insures stability of the triplex PNAS.

18, the Triplex Lock Flap hairpin oligo with reverse polarity to produce a DNA/RNA heteroduplex to help stabilize the PNAS and render it EXO III resistant (requirement for 3' end)

19, Binding partner for the biochemical hook, for example, the solid support is coated with protein called streptavidin which has high affinity for the biochemical hook biotin

20, the solid supports can be any supports commonly used in assays such as test tube walls, magnetic beads or microtiter plate wells

21, the secondary signal amplification probe Poly dT (3'- 5') that forms the Duplex Reporter.

22, the secondary signal amplification probe Poly dT (5'- 3') that forms the Triplex Reporter.

23, the label, DIG, on the reporter probes can be detected by reaction with the anti-DIG-enzyme conjugate.

24, the signal amplification Poly dT probes

25, a spacer or linker molecule

26, the endonuclease resistant hairpin

27, the Exonuclease resistant structure

mRNA TPA: Gel Format

The gel format for mRNA TPA essentially follows the test tube format as previously discussed. The three levels of specificity included are one, triplex PNAS formation, two, restriction endonuclease treatment combined with EXO III degradation, and three, migration in a non-denaturing gel to a predictable Rf.

mRNA TPA gel format is presented in general, in Figure 7A and 7B with the following modifications:

Step I: Isolation of mRNA by any method known by those skilled in the art. A polypyrimidine target site is preferable. See Fig. 7A 1)

Step II: Design and Hybridization of a TFO (ds DNA Hairpin) with the triplex lock. The combination of the target sequence and the ds DNA hairpin form the PNAS (protected nucleic acid sequence). (First level of specificity: Triplex PNAS Formation) See Fig. 7A 2)

Step III: Restriction endonuclease destruction of unbound ds DNA hairpin probes. Subspecies specific mRNA/Hairpins complex (PNAS) survives the treatment with the exonuclease. Hairpins not protected by the target are cut at the restriction endonuclease site, followed by degradation by EXO III completely destroying all non-specific hairpins. Both enzyme treatments can be considered together as a single level of specificity. See Fig. 13 A 3)

Step IV: EXO III treatment to degrade the, unprotected, restricted hairpin (newly exposed 3' ends) (Second Level of Specificity: RE and EXO treatment) See Fig. 7A 4)

Step V: Anchor the PNAS to a solid support through the biotin-streptavidin interaction. See Fig. 7A 5)

Step VI: Wash to remove non-target mRNA enzyme and restricted probe.

Step VII: Hybridize the Poly A region with the Poly dT signal amplification pairs, conjugated with DIG at least every 5 bases under conditions to form the triplex Poly PY • Poly PU • Poly PY. See Fig. 7A 6)

Step VIII: Wash to remove unbound probe.

Step IX: Dissociate PNAS from the solid support structure without compromising the PNAS structure

Step X: Electrophorese to a predictable Rf (again, only the relative migration distance is important. (Third Level of Specificity: Gel Migration to a predictable Rf.)

Step XI : Identify target band position in the gel by addition of UV fluorescent intercalating dyes such as ethidium bromide or by addition of the anti-DIG-enzyme conjugate to the gel after washing and placement of the gel in a chromogenic substrate solution (incubate to allow color development to access band Rf (location). Other detection methods are contemplated as part of the methods of the present invention.

mRNA RFTA: Test Tube Format

The Restriction Fragment Target Assay (RFTA) can be configured for sensitive mRNA subpopulation analysis. Figure 8A to 8B represents the mRNA RFTA test tube format with the following steps:

Step I: Isolation of mRNA by any procedure known to those skilled in the art. See Fig. 8A 1)

Step II: Hybridize with a primary probe with a Poly dA reporter tail. The target sequence (mRNA subspecies specific) is of variable length and usually greater than 12-15 nucleotides varying in all 4 bases (A,T,G,C) or polypurine or polypyrimidine, both of which vary in 2 bases (A,G) and (T,C) respectively. This probe may have a 5' cap or a 3'cap. (First Level of Specificity) See Fig. 8A 2)

Step III: Hybridize with a biotinylated secondary capture probe of variable length (preferably greater than 12-15 bases) (Second Level of Specificity). The capture probe has a means for capturing the complex, for example, using biotin as the capture molecule that is bound by its binding partner, streptavidin. See Fig. 8A 3)

Step IV: Treatment with exoribonuclease (3'- 5' Degradation of ssRNA) See Fig. 8B 4)

Note: The mRNA target is degraded from the 3' end up to the internal target nucleotide sequence where secondary structure is initiated (point at which the duplex forms). The target sequence can vary in the four bases (A,T,C,G) or be a polypyrimidine or polypurine sequence. The length of this target is variable, however, generally stated as being 18 nucleotides in length. Again, nuclease treatment adds to the specificity of the process; and can be assessed as an additional specificity level due to the complete destruction of all non-specific mRNA molecules, 3' poly A reporter regions. (Third Level of Specificity)

Step V: Target Capture: Addition of a solid support, e.g., magnetic beads coated with a binding partner of the capture molecule, e.g., streptavidin. See Fig. 8B 5)

Step VI: Wash to remove debris principally from non-target containing mRNA.

Step VII: Add secondary reporter probes Poly dT (3'- 5' and 5'- 3') forming a Triplex Reporter probe complex (Poly PY • Poly PU • Poly PY) See Fig. 14 B 6)

The Poly dT 3'- 5' and 5'- 3' are 40 mer in length and up to six probes bind to the 5' Poly A end of the primary probe ~120 mer. The DIG is conjugated at every fifth base, therefore 8 DIG molecules are added to the PDTP complex by the binding of each of the Poly dT-DIG probes (up to 48 per triplex reporter region).

Although the Poly dA reporter region must first bind poly dT of opposite (antiparallel) polarity to form the duplex and next the poly dT must bind with parallel polarity to the poly dA (Watson strand) with Hoogsteen's bonding to form the triplex, it is by convention necessary to evoke no level of specificity herein due to the fact that all mRNAs have Poly A 3' ends.

Step VIII: Wash to remove unbound probe

Step IX: Add anti-DIG-enzyme conjugate (48 conjugate molecules bound per reporter triplex)

Step X: Wash to remove unbound conjugate under conditions that stabilize triplex formation.

Step XI: Add a chromogenic substrate which together with the enzyme present on the conjugate produces a colored soluble substrate (to be quantified at the appropriate wavelength.

Both qualitative and quantitative results can be obtained as is the case with all test tube assays involving TPA, RFTA, and CPA processes.

The numerical designations for mRNA RFTA test tube format in Figures 8A and 8B are as follows:

1, the target sequence of variable length that varies in 4 bases or in 2 bases, such as a polypurine or polypyrimidine rich region. In general, the length is preferably. 18 bases to insure specificity in binding.

2, the 5' encoding end of the mRNA molecule

3, the non-specific mRNA region

4, the 3' Poly A mRNA region (approximately 250 bases long)

5, the 3' end of the reporter probe capped by any method to render it resistant to nuclease attack, or a 5' end that is nuclease resistant.

6, the subspecies specific mRNA molecule

7, the primary probe consisting of a 5' end varying in 4 or 2 bases (as discussed) of variable length and the 5' or 3' capped Poly dA end also of variable length of approximately ~120 bases in length. Note the 3' end may be capped to render it nuclease resistant without a reversal of oligo polarity.

8, normal hydrogen bonding stabilizing the DNA/RNA heteroduplex formed

9, the Partial Duplex Target Probe Complex (PDTP Complex)

10, the Poly dA reporter region of the primary probe of variable length (this embodiment ~120 bases long)

11, the secondary biotinylated capture probe of variable length, usually 18 nucleotides.

12, the biochemical hook, a biotin molecule

13, the solid support in this case a magnetic bead coated with streptavidin.

14, streptavidin has high affinity for biotin and facilitates attachment of the target to a solid support.

15, the Poly dT amplification probe, 40 mer, conjugated with DIG at every fifth base a minimum of 8 DIG per probe molecule.

16, the Poly dT (3'-5') format forms a duplex with the Poly dA region of the primary probe (5'-3'); antiparallel orientation and hydrogen bonded.

17, the Poly dT (5'-3') format forms a triplex with the Poly dA region of the primary probe (5'-3'); parallel orientation and Hoogsteen bonded.

18, Hoogsteen Bonding of the polypyrimidine dT (5'-3') and Poly A (5'-3')

19, the reporter triplex Poly dT • Poly dA • Poly dT, providing 48 DIG molecules per target region

20, Label molecule

21, Means for attaching the capture molecule such as a covalent bond or linker.

mRNA RFTA: Gel Format

The RFTA process can be configured for sensitive mRNA subpopulation analysis. Figure 8A and 8B presents the mRNA RFTA gel format in general, with the following modifications.

Step I: Isolation of mRNA by any procedure known to those skilled in the art. See Fig. 8A 1)

Step II: Hybridize with a primary probe with a Poly dA reporter tail. The target sequence (mRNA subspecies specific) is of variable length and usually greater than 12-15 nucleotides varying in all 4 bases (A,T,G,C) or polypurine or polypyrimidine, both of which vary in 2 bases (A,G,) and (T,C) respectively. (First Level of Specificity) See Fig. 8A 2)

Step III: Hybridize with biotinylated secondary capture probe of variable length (greater than 12-15 bases) (Second Level of Specificity) See Fig. 8A 3)

Step IV: Treatment with exoribonuclease (3'→5' degradation of ssRNA) See Fig. 14 B 4)

The mRNA target is degraded from the 3' end up to the internal target nucleotide sequence. This sequence can vary in the four bases (A,T,C,G) or be a polypyrimidine or polypurine sequence. The length of this target is variable, however, generally being 18 nucleotides. Again, nuclease treatment adds to the specificity of the process; and may be assessed as an additional specificity level due to the complete destruction of all non-specific mRNA molecules 3' prime end poly A reporter regions. (Third Level of Specificity)

Step V: Target Capture: Addition of Magnetic Beads coated with streptavidin. See Fig. 8B 5.

Step VI: Wash to remove debris principally from non-target containing mRNA.

Step VII: Add secondary reporter probes Poly dT (3'-5' and 5'3') forming a Triplex Reporter probe complex (Poly PY • Poly PU • Poly PY) See Fig. 8B 6)

The Poly dT 3'-5' and 5'-3' are 40 mer in length and up to six probes bind to the 5' Poly A end or the 3' capped end of the primary probe. The DIG is conjugated at every fifth base, therefore 8 DIG molecules are added to the PDTP complex by the binding of each of the Poly dT-DIG probes (up to 48 per triplex reporter region.

Although the Poly dA reporter region must first bind poly dT of opposite (antiparallel) polarity to form the duplex and next the poly dT with parallel polarity must bind to the poly dA with Hoogsteen's bonding to form the triplex, it is by convention necessary to evoke no level of specificity herein due to the fact that all mRNAs have Poly A 3' ends.

Step VIII: Wash to remove unbound probe

Step IX: dissociate the PDTP complex from the solid support and run it on a non-denaturing gel and determine the Rf by addition of the anti-DIG enzyme conjugate and a chromogenic substrate (precipitate forming) or by use of intercalating dyes such as ethidium bromide, direct dye fluoresence, with which UV or other directed fluorescence can pinpoint the band location so Rf can be measured. (Third Level of Specificity: Gel Migration to a predictable Rf .)

Both qualitative and quantitative results can be obtained as is the case with all test tube and gel assays involving TPA, RFTA, and CPA processes.

DNA TPA: TEST TUBE FORMAT

DNA TPA tube assay is presented in Figure 9A and 9B with the following steps:

Step I: DNA is isolated by any method known by those skilled in the art. See Fig. 9A 1)

Step II: Endonuclease restriction of the target and adjacent upstream and downstream regions. In effect, each endonuclease should confer one level of specificity (2 enzymes-2 levels of specificity); however, since endonuclease restriction is not necessary to see a result (genomic DNA has nicks) these one or two levels are not accessed. Furthermore, since the gel application requires proper sizing of the target, a level of specificity can be assessed, therein, and can be taken at the Restriction Endonuclease stage or at the gel migration to a predetermined R_f stage. See Fig. 9A 2)

Step III: Hybridize a Triplex Forming Oligonucleotide (TFO) (First Level of Specificity) See Fig. 9A 3)

The capture molecule, Biotin or any other specific binding partner, may be located on the TFO or the capture probe. If additional levels of signal amplification are required, then the capture molecule, biotin, is located on the TFO and two reporter probes may be used.

Step IV: Digestion with EXO III or other suitable enzyme that will generate the PNAS 5' Tails See Fig. 9A 4)

In DNA TPA, the probe (TFO) protects the double strand target from nuclease degradation, whereas in mRNA TPA the duplex probe (hairpin or RP-TFO "nonhairpin") protects the triple stranded target from nuclease degradation.

Step V: Hybridize with a biotinylated capture probe (Second Level of Specificity) See Fig. 9A 5)

Step VI: Capture by addition of streptavidin coated solid support or the specific binding partner of the above molecule attached to TFO or capture probe. See Fig. 9B 6)

Step VII: Wash to remove unbound reporter probe

Step VIII: Hybridize with the reporter probe 3' end (capped or uncapped) Poly dA (~120 mer) and 5' end complementary to the reporter region (18mer). (Third Level of Specificity) See Fig. 9B 7)

Step IX: Wash to remove unbound reporter probe

Step X: Add a secondary signal amplification probe Poly dT (3'-5' and 5'-3') with labels, forming a triplex reporter Poly Py • Poly Pu • Poly Py. As previously discussed, the triplex is held together by the hydrogen bonding of the w and c strands as well as the Hoogsteen bonding between the third strand and the purine watson (w) strand. See Fig. 9B 8)

Another embodiment of the DNA TPA assay comprises use of 2 reporter regions as the PNAS tails. See Fig. 9B 9. Both of the tails may function as reporter regions. Preferably, the reporter regions have sequences. See Fig. 9B 9. Using one reporter probe provides:

- Poly dT (40 mer) 3'-5' and 5'-3'
- up to 6 Poly dT probes bind to the Poly dA end of the primary probe
- DIG is conjugated at least at every fifth base, therefore 6 Poly dT probes each with 8 DIG present
- up to 48 opportunities for binding of the anti-DIG-enzyme conjugate

Having two reporter regions presents up to 96 opportunities for binding the signal producing enzyme or molecule producing signal. Ultimately, this represents a 100 fold increase in sensitivity. In such an embodiment the TFO could be the capture probe (conjugated with a capture molecule such as biotin) allowing use of a double reporter.

Step XI: Wash to remove unbound signal amplification probes

Step XII: Add the anti-DIG antibody-enzyme conjugate.

Step XIII: Wash to remove unbound conjugate.

Step XIV: Add a chromogenic substrate together with enzyme interaction yields a soluble colored molecule.

This result may be qualitative or quantitative.

Numerical Designations indicated for DNA TPA Test Tube Format for Figures 9A and 9B follow:

1, DNA target polypurine and polypyrimidine rich region

2, restriction site one near the target (distance may vary greatly)

3, restriction site two near the target (distance may vary greatly)

4, the triplex (TFO/Target Complex) PNAS conferring nuclease resistance to the ds DNA target.

5, the biochemical hook, biotin

6, the PNAS/Tails structure

7, the capture probe complementary to the capture tail region

8, the reporter tail region complementary to a region on the reporter probe.

9, the reporter probe with the 3' Poly dA region and a cap and whose 5' end binds to the reporter region

10, the 3' end may be capped to provide nuclease resistance of the target or nay be converted to a 5' end by reversal of the polarity of the oligonucleotide.

11, the solid support (magnetic bead)

12, the protein, streptavidin, that binds to biotin with high affinity.

13, the signal amplification probe Poly dT (pair).

14, the Poly dT (5'-3') forming the duplex Poly dA(3'-5') • Poly dT(5'-3').

15, the Poly dT (3'-5') forming the triplex Poly dT • Poly dA (3'-5') • Poly dT

16, normal hydrogen bonding in the duplex region

17, Hoogsteen's Bonding between the 3rd strand of Poly dT conjugated with DIG and the (w) strand of the duplex.

18a, the reporter probes bound to the PNAS structure to amplify the signal (Poly dA region) may be doubly bound to the PNAS (2 Poly dA regions per target. In the two reporter embodiment, the biochemical hook may be attached to the TFO

18b, the PNAS tail

19, the Reporter Triplex Poly dT • Poly dA • Poly dT

20, the label DIG

21, the TFO (Triplex Forming Oligonucleotide)

22, the reporter probe

23, means for attaching the capture molecule, e.g. a covalent bond or linker.

DNA TPA: GEL FORMAT

DNA TPA gel assay is presented in Figures 9A and 9B with the following modifications: Step I- Step VII comprise the same method steps as Steps I-VII in the DNA TPA Test Tube Format, described above.

Step VIII: Hybridize with a probe consisting of a 5' end or 3' end and a capped Poly dA (~120 mer) and 5' end complementary to the reporter region. (Third Level of Specificity) See Fig. 9B 7)

The poly dA segment length was chosen by the longest polynucleotide that could be synthesized at a reasonable cost. Alternate cost efficient methods of longer oligonucleotide production would support increased test sensitivity.

Step IX: Wash to remove unbound probe

Step X: Add a secondary signal amplification probe Poly dT (3'-5' and 5'-3') forming a triplex reporter Poly Py • Poly Pu • Poly Py. As previously discussed, the triplex is held together by the hydrogen bonding of the w and c strands as well as the Hoogsteen Bonding between the third strand and the purine watson (w) strand. See Fig. 9B 8)

Another embodiment of the DNA TPA gel format comprises use of 2 reporter regions as the PNAS tails. See Fig. 9B 9. Both of the tails function as reporter regions. In this case, the capture function may be accomplished by the TFO with a capture molecule. Preferably, the reporter regions have different sequences. See Fig. 9B 9. Each reporter probe comprises:

- Poly dT (40 mer) 3'-5' and 5'-3'
- Up to 6 Poly dT probes bind to the Poly dA end of the primary probe
- DIG is conjugated at least at every fifth base, therefore 6 Poly dT probes each with 8 DIG present represent up to 48 labels being attached to the target.

Step XI: Wash to remove unbound probe

Step XII: Dissociate PNAS from the magnetic bead structure without compromising the PNAS structure

Step XIII: Electrophorese to a predictable Rf. Again, only the relative migration distance is important. (Fourth Level of Specificity)

Step XIV: Identify target band position in the gel. Currently used methods include addition of UV fluorescent intercalating dyes such as ethidium bromide or by addition of the

anti-DIG-enzyme conjugate to the gel and after washing each placement of the gel in a chromogenic substrate solution (incubate to allow color development to assess band Rf (location).

DNA RFTA: TEST TUBE FORMAT

An embodiment of a DNA RFTA test tube assay format is presented in Figures 10A to 10B. The methods for this assay comprise the following steps.

Step I: Isolate genomic DNA by any method known to those skilled in the art and select two restriction endonuclease sites that will restrict the target region. See Fig. 10A 1)

Step II: Endonuclease restriction of the target, using one or two enzymes, or other methods of restricting DNA, yields a double stranded target region. See Fig. 10A 2)

The levels of specificity imparted by the use of 1 or 2 restriction enzymes as previously presented cannot be assessed in the tube format of the assay.

Step III: Denature the duplex target and the remaining genomic DNA and hybridize with the primary probe. (First Level of Specificity) See Fig. 10A 3)

Step IV: Hybridize with the biotinylated capture probe, or any capture probe with a capture molecule attached (a primary probe). (Second Level of Specificity) See Fig. 10A 4)

Step V: Hybridize with a capped reporter probe consisting of a 3' end capped or 5' end Poly dA region and a 5' end complementary to the reporter region (reporter tail of PDTP Complex) (the secondary probe). The Poly dA region is preferably approximately 120 nucleotides in length; however, the length and number of nucleotides may vary. (Third Level of Specificity)

Step VI: Capture the PDTP Complex on a solid support, for example a magnetic bead. See Fig. 10B 5)

Step VII: Wash to remove unbound primary and secondary probes.

Step VIII: Hybridize with the poly dT labeled signal amplification probe pair-Poly dT (3'-5') and (5'-3')-forming a triplex reporter structure Poly Py • Poly Pu • Poly Py with the poly dA region of the primary probe. See Fig. 10B 6)

Up to 6 of the Poly dT probes can bind to the Poly dA reporter region forming duplex and up to 6 additional poly dT to form the triplex Poly dT (3'-5') binds antiparallel to the Poly dA forming a duplex structure, stabilized by hydrogen bonding

Poly dT (5'-3') binds parallel to the Poly dA forming a triplex structure, stabilized by Hoogsteen's bonding. For labeling purposes, an example of DIG attached probes is presented. Each Poly dT (40 mer) has DIG conjugated to at least every fifth base. Therefore, up to 48

opportunities are available to affix a label, such as signal amplification enzyme to the target labels have been discussed previously. See Fig. 10B 7)

Step IX: Wash to remove unbound amplification probe

Step X: add the antibody-enzyme conjugate (IgG anti-DIG/enzyme)

Step XI: Wash to remove unbound conjugate.

Step XII: add a chromogenic substrate which upon enzyme interaction produces a soluble product. Quantitate by measurement of absorbency or any other detection method.

The numerical designations presented for DNA RFTA test tube assay format in Figures 10A to 10B are as follows:

1, the target site

2, the restriction site number one

2', the restriction site number two

3, the capture region which is located on the watson strand of the denatured target that does not bind to the primary probe

3' the reporter region which is the 5' end of the primary probe that does bind to the target

4, the primary probe that binds to only a section of the target region.

5, the capture probe (biotinylated)

6, the biochemical hook, biotin

7, the reporter probe is an oligo of varying length that has a 5' end complementary to the reporter region of the PDTP Complex and the other end can be a 3' capped end or 5'end (reverse polarity) that is nuclease resistant and that provides a Poly dA region, that is approximately 120 nucleotides, to allow detection of the PDTP complex.

8, the section of primary probe complementary to the reporter region

9, Poly dA section of the primary probe that will allow formation of the Poly dT • Poly dA • Poly dT reporter triplex for detection of the PDTP Complex

10, the PDTP complex

11, the solid support which in this embodiment is a magnetic bead

12, streptavidin is a protein that binds with very high affinity to the biotin (biochemical hook on the capture probe)

13, the Poly dT signal amplification probes (3'-5') and (5'-3')

14, Poly dT (5'-3') binds antiparallel to the poly dA strand forming a stable hydrogen bonded duplex and delivers as many as 24 DIG molecules to the PDTP complex

15, Poly dT (3'-5') binds parallel to the poly dA strand forming a stable hydrogen bonded duplex and delivers as many as 24 DIG molecules to the PDTP complex

16, Hoogsteen Bonding (lesser hydrogen bonds) that allow triplex formation

17, Hydrogen Bonding (normal duplex DNA or DNA/RNA or RNA/RNA interactions

18, the DIG (DIGOXYGENIN) is the molecule used to attach the DIG-anti-enzyme conjugate, the first step in signal development.

19, the Reporter Triplex

20, means for attaching the capture molecule

DNA RFTA: GEL FORMAT

An embodiment of a DNA RFTA gel assay format is presented in Figures 10A and 10B with the following modifications. The methods for this assay comprise the following steps.

Step I: Isolate genomic DNA by any method known to those skilled in the art and select two restriction endonuclease sites that will restrict the target (size the target or larger). See Fig. 10A 1)

Step II: Endonuclease restriction of the target (one or two enzymes) results in the production of a double strand target region. See Fig. 10A 2)

The levels of specificity imparted by the 1 or 2 restriction enzymes as previously presented cannot be assessed in the tube format of the assay (while providing one level of specificity in the gel format that is assessed after the gel has run and the PDTP Complex has migrated to a predetermined point).

Step III: Denature the duplex target and the remaining genomic DNA and Hybridize with the primary probe. (First Level of Specificity) See Fig. 10A 3)

Step IV: Hybridize with the biotinylated capture probe (Second Level of Specificity) See Fig. 10A 4)

Step V: Hybridize with the capped reporter probe consisting of a 3'end capped or 5' Poly dA region and a 5' end complementary to the reporter region (reporter tail of PDTP complex). The Poly dA region is ~120 mer; however, it may vary in length. (Third Level of Specificity)

Step VI: Capture the PDTP Complex on a solid support (magnetic bead). See Fig. 10B 5)

Step VII: Wash to remove unbound primary and secondary probes.

Step VIII: Hybridize a secondary reporter probe pair, Poly dT (3'-5') and (5'-3'), forming a triplex reporter structure Poly PY • Poly PU • Poly PY with the poly dA region of the primary probe. See Fig. 10B 6)

Up to 6 of the Poly dT probes can bind to the Poly dA reporter region forming a triplex Poly dT (5'-3') binds antiparallel to the Poly dA forming a duplex structure, stabilized by

hydrogen bonding. Poly dT (3'-5') binds parallel to the Poly dA forming a triplex structure stabilized by Hoogsteen's bonding.

Each Poly dT (40 mer) has DIG conjugated to at least every fifth base. Therefore, up to 48 opportunities are available to affix a signal amplification enzyme to the target. See Fig. 10B 7)

Step IX: Wash to remove unbound signal amplification probe

Step X: Dissociate the modified PDTP Complex from the magnetic beads with the complex remaining intact.

Step XI: Run a non-denaturing electrophoretic gel to a predetermined Rf, (Fourth Level of Specificity)

Step XII: Add the antibody-enzyme conjugate to the gel or intercalating dyes such ethidium bromide to locate the target position in the gel.

Step XIII: Add a chromogenic substrate to the gel which together with enzyme interaction yield an insoluble colored precipitate.

Reverse Dot Blot Formats of TPA and RFTA

Another embodiment of the present invention, assay formats called Reverse Dot Blot assays, provide increased sensitivity over the gel format while supporting rapid testing of multiple specimens with the capability of multiplexing for identification of multiple targets in a single specimen.

The dot blot assay format was developed as a tool for analyzing DNA from a specimen and utilizing a probe to signify the presence of the specific target. Quantitative dot blots may be possible to configure if appropriate standards are employed; however, dot blot assays usually provide qualitative results.

A disadvantage of the classical dot blot assay format was that one source of nucleic acid, such as DNA, would have to be placed on separate membranes to allow hybridization with multiple probes. This increased the costs of dot blot assays and was a problem when there was a small amount of source nucleic acid. A variation of the dot blot format is the reverse dot blot format. This reverse format corrected the shortcomings of the dot blot format by permitting multiple probes to be applied to a single membrane and exposing that membrane to the source nucleic acid.

In this method, one or any number of oligonucleotide probes is tailed with poly (dT) on the 3' end, preferably using terminal transferase and and is then immobilized on a memberane,

preferably by UV crosslinking to a nylon membrane. The probe and the poly dT tail may be of any length. Preferably the length of the poly dT tail is 100-200 nucleotides.

Currently, there are many different types of membranes available for this type of assay. A preferred type of membrane is the nylon membrane, of which two basic types of nylon membranes are commercially available, unmodified and charge modified nylon. Both types of membranes are preferred. The charge modified nylon is more preferred due to its positively charged surface providing enhanced capacity to bind the highly negative nucleic acids and other molecules, such as chromogenic insoluble substrates.

A dot blot filtration manifold is used to produce the nylon strip carrying the various oligonucleotide probes. Any strip production process known is acceptable.

In general, the methods of use comprise inserting a precut nylon membrane into the dot blot manifold at which time the membrane is wet with DNA grade ultrapure water (DNA H2O). A volume of probe solution of any concentration, usually providing 4 picomoles of oligonucleotide probes in buffer (5X SSPE), tailed with poly (dT) using terminal transferase, is added to individual wells in pre-determined ordered sequence on the membrane. The vacuum manifold is then turned on and the oligonucleotide probe solutions are filtered through the nylon membrane (usually 0.2 to 0.45 micron pore size) with essentially all of the probe remaining bound to the highly positive nylon membrane. Each manifold well is washed by adding 100 microliter of TE or similar low salt buffer to the wells and applying the vacuum. Next, the damp membrane is removed from the manifold, and is irradiated with short wave-length UV light 254 nm at a dose rate of 1.5 J/sq. cm. (dry membranes may be used; however, irradiated at a dose rate of 0.15 J/sq. cm.) to facilitate covalent bonding of the probe to the membrane. The UV causes the T-tail on the capture probe to form thymine covalent crosslinks with the nylon membrane. Usually, a series of preliminary experiments are necessary to determine empirically the amount of irradiation necessary to produce the maximum hybridization signal. The fixed membrane is washed to remove unbound probe by any method known to those skilled in the art. To accomplish this the membrane is usually incubated for at least 30 minutes at 55 degrees C in 5X SSPE, 0.5% SDS.

If the membrane is not used immediately for specimen analysis, it should be wrapped loosely, separated by filter paper sheets, in aluminum foil and stored in vacuo or under dessication at room temperature.

Hybridization conditions for the membrane are known to individuals skilled in the art. Consideration should be directed toward, the buffer and temperature used, the volume of solvent and length of hybridization time, the degree and method of agitation (continuous shaking or

stationary), use of membrane blocking agents to block the non-specific attachment of the probe to the surface of the solid matrix in the prehybridization stage, use of polymeric compounds, such as dextran sulfate and polyethylene glycol that increase the rate of reassociation of nucleic acids, and the stringency of washing following the hybridization.

The hybridization solution (5X SSPE, 0.5% SDS) is prewarmed to 55 degrees C before use. When nylon membrane is used, blocking agents are often omitted from the prehybridization and hybridization solutions, since high concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is important to consider when the oligonucleotide probes are less than 100 nucleotides in length.

To maximize the rate of annealing of the probe with its target, hybridization is usually performed in solutions of high ionic strength (5X or 6X SSPE) at a temperature that is 20-25 degrees C below the melting temperature (Tm) of the target/probe complex.

The sample nucleic acid is added to the hybridization solution. For double stranded nucleic acids, the sample must first be denatured, for example, by heating at 95 degrees C for 5 minutes, then placing on ice rapidly to avoid reannealing.

Assays of the present invention, including but not limited to TPA, RFTA and CPA, yield the resulting target/probe complex structure (PNAS or PDTP Complex) which possesses a ss tail (capture region) to hybridize to a specific ss capture probe with a poly (dT) tail bound and covalently linked to the nylon membrane.

Next, the hybridized membrane is washed with the most stringent washing conditions, preferably a combination of temperature and salt concentrations that is approximately 12-20 degrees C below the calculated Tm of the hybrid and a lower salinity wash. In order to minimize problems with high background signal, it is preferable to hybridize for the shortest possible time using the minimum amount of probe.

In general, one can place the membrane strips in a hybridization plate trough, add 2 to 3 milliliters of hybridization solution, and add the PNAS/tail and PDTP complexes in a minimal volume of buffer, preferably 20-100 microliters, which is designed to stabilize the complex and incubate for the optimimal time and temperature. These conditions will vary depending on the AT and GC content of the nucleic acids. The hybridized membrane is washed as previously discussed. Note the hybridization temperature can range from 5 to 20 degrees C lower than the predicted Tm of the oligonucleotide in 1 M salt. The stringency of the wash is increased by either lowering the salt concentration or increasing the hybridization temperature. A typical wash solution, generally, has less salt and the wash temperature is 5 degrees C lower than that of the hybridization conditions with moderate shaking.

Next, in the following TPA and RFTA Reverse Dot Blot formats a reporter probe (approx. 40 nucleotides) posessing an attached label is hybridized to the membrane bound PNAS and PDTP complexes and the membrane then washed once or twice under stringent conditions, again at a lesser salt concentration (1XSSPE plus 1% Triton X-100) and at a temperature 5 degrees C less than the hybridization temperature for 10 minutes with moderate shaking.

Another signal amplification comprises use of the Poly dA tailed reporter probe. Such a probe comprises a 5' end complementary to the reporter region of the PNAS or PDTP complexes with a 3' capped end or 5' end Poly dA approximately 120 mer. Hybridization of the PNAS or PDTP complex with the probe would provide the 3' Poly dA tail 120 mer of the complex to generate signal amplification via reporter triplex formation by subsequent hybridization with the labeled poly dT signal amplification probes (a pair), Poly dT (5'-3') and Poly dT (3'-5') and the polyamine hairpin probes conjugated with the identical label. The signal amplification strategy has been previously discussed.

In some embodiments, biotin is the label attached to the probe. In those instances, streptavidin-HRP (horseradish peroxidase) conjugate in the buffer (1XPBS, 100mM NaCl, 5% Triton X-100 is added to the fully hybridized wet membrane, and incubated for 10 minutes with moderate shaking. Adapt the membrane to the chromogen producing reagents by incubating the membrane in buffer B (buffer A, 1 M urea, 1% dextran sulfate) for 5 minutes at room temperature, with moderate shaking.

The final step of the assay is color production of the insoluble colored precipitate from the chromogen selected. Tetramethylbenzidine, etc., or any known to those skilled in the art. The particular steps depend on the label selected that is attached to the probe.

After hybridization and washing of the nylon membrane, the surface should contain complexes of the T-tailed capture probe/PNAS tail structure/reporter probe/label. None of these four moieties should non-specifically hybridize to non-specified sections of membrane due to the stringent hybridization conditions and repeat washings to insure a low signal background.

The final step in the reverse dot blot format is the visualization of the bound complexes. For example, the T-tailed capture probe/PNAS tail/Biotinylated Reporter probe-avidin enzyme complex is visualized by addition of a chromogen that produces an insoluble colored precipitate upon interaction with enzyme molecules in the target complex. The highly negatively charged chromogen readily binds to the highly positively charged membrane.

The exact chromogen can be any known to those skilled in the art; usually, in this case, producing an insoluble colored precipitate when acted on by an enzyme. (a soluble colored

substrate may also be produced; however, in the reverse dot blot format the colored solid precipitate is more appropriate for use).

DNA TPA Reverse Dot Blot Format

An embodiment of a DNA TPA Reverse Dot Blot assay format is presented in Figures 11A and 11B. The methods for this assay comprise the following steps.

Step I: Isolation of the sample nucleic acid, for example, high molecular weight genomic DNA. This procedure can be any method known by those skilled in the art.

Step II: Sample preparation comprises shearing or restriction cutting of the sample nucleic acid, releasing the target segment. The sample nucleic A segment including the target and an upstream region (variable length) and a downstream region (variable length) may be restricted by any method known to those skilled in the art. This may be achieved by endonuclease restriction site analysis of the target and adjacent regions resulting in the selection of a restricted fragment that is produced by use of one or two restriction endonucleases that have no recognition site within the target and upstream/downstream region, resulting in a restricted segment of DNA. The goal of multiplex identification of a number of targets in the sample, simultaneously, necessitates the similar evaluation of each target region.

One method for cutting or restricting the sample is to use the BU cutters of the present invention.

Another approach to the restriction of multiple target sites, also necessitates target region inspection of all target sites and selection of a frequent base cutter restriction endonuclease (4 base cutter) that is characterized as not having a site located within any target or adjacent upstream and downstream regions and as providing a minimal and maximal length of the upstream and downstream regions.

Another approach for the non-enzymatic restriction of targets without the use of the restriction endonucleases is based on the controlled and unbiased shearing of DNA. The Point-Sink Shearer (P+S), is a machine that performs controlled and unbiased shearing of DNA. Results generated by the hydrodynamic point-sink shearing method (Oefner, 1996) indicate that ninety percent of sheared DNA fragments fall within a two-fold size distribution that is high reproducible. Each iteration (repeat round) of sample shearing provides a predictably smaller and smaller median size (Kb) fragment.

In a diagnostic assay where targets are plentiful, the random shearing of the genomic DNA would provide a range of DNA segments containing the entire complete target and partial targets produced by shearing of the target itself. The plentiful abundance of the target present

assures that sufficient complete target DNA segments (Kb) will be present for Haystack Processing and subsequent target identification. This would not configure to a quantitative assay, just a good qualitative assay.

The variable length capture tails (5') generated by either the frequent restriction endonuclease cutter or by controlled shearing use and ExO III treatment can be handled by requiring that the capture probe have complementarity to a region closely flanking the protected target on the 5' tail. Any capture tail of variable length attached to a PNAS can bind to a probe with a capture sequence closely flanking the PNAS. The remaining segment of the 5' tail would be unimportant to the end result.

Step III: Add a unique Triplex Forming oligonucleotide for each target region to be analyzed. The rules of design of the TFOs are known to those skilled in the art. Generally, it is preferable that the target is a polypurine-polypyrimidine rich region. Triplexes can be formed with other types of regions, and such triplexes can be stabilized by a variety of techniques, for example, using modified bases or stabilizing molecules or cross-linking methods. For enhanced stability at a neutral pH, the TFO will be a polypyrimidine oligonucleotide with the cytosine bases replaced by 5'-methylcytidine. (First level of specificity)

The sample DNA now exists as segments of DNA (Kb or smaller), containing the protected target. The triplex length in this embodiment is preferably 18 bases.

Step IV: Sample DNA is digested with Exonuclease III (EXO III) or other suitable enzyme under optimal conditions for enzyme activity and triplex stability known to those skilled in the art.

After incubation, the protected triplex target, flanked by 2 dsDNA regions, becomes converted to a triplex protected target and two 5' flanking tails (ssDNA). When EXO III is used, it degrades blunt-ended DNA in the direction of 3'→5' on the Watson and Crick strand, but stops at the triplex, rendering it exonuclease resistant. This structure will hereafter be referred to as Protected Nucleic Acid Sequence with two 5' Tails (PNAS/tails). One 5' tail is a capture region and the other 5' tail is a reporter region, yielding places for binding a capture and a reporter probe, respectively.

The sample processing is now complete and the target probe complexes in the the sample are now ready for reverse dot blot testing.

Step V: Production of the probe bound nylon membrane. The nylon membrane dot blot strip is prepared by methods known to those skilled in the art, preferably using the methods previously discussed. The strip contains specific areas that are composed of the different T-tail

probes bound to the nylon membrane, and negative and positive controls for each of the targets being identified. See Fig 11A 1)

Step VI: Wash the membrane prior to hybridization to remove unbound capture probe

Step VII: PNAS hybridization to the nylon membrane with the bound capture probes. Target (PNS/Tails) presence will allow the PNAS/tail structure, namely the tail region functioning as the capture region, to hybridize to the capture probe attached to the nylon membrane. (Second level of specificity) See Fig 11A 2)

Step VIII: Hybridization of a reporter probe to the PNAS/tails 5' reporter tail. In this example, this probe has Digoxygenin (DIG) molecules attached at various sites throughout the probe. Other labels can be used with the present invention and the invention is not limited to the examples given herein. Target identification is produced by the interaction of the DIG with the anti-DIG-enzyme conjugate and subsequent exposure to a chromogenic substrate. See Fig. 11A 3)

Increased signal amplification can be provided by a novel method called triplex reporter formation using the labeled poly dT (5'-3' and 3'-5') signal amplification probes. Each poly dT probe is bound in at least every fifth base with DIG (to bind the signal producing anti DIG-Enzyme conjugate. The Poly dT (3'-5') produces a stable duplex structure with a poly dA (5'-3') region on the reporter probe. At this point, up to 24 DIG molecules can bind to the PNAS complex.

If additional signal amplification is required, the Poly dT (5'-3') can be added under conditions favoring triplex formation; a reporter triplex is formed and up to an additional 24 DIG molecules (48 total) can be attached to the PNAS complex. Another variation on the scheme would be to attach a self-complexing probe to the reporter probe that would participate in a probe complex formation on the reporter probe and additional local DIG accumulation. (Third level of specificity)

Step IX: Wash to remove unbound probe

Step X: Hybridize the Poly dA reporter region of the DNA target with a poly dT signal amplification probe pair, both (40 mer), Poly dT (5'-3') and Poly dT (3'-5'). These are conjugated with DIG at least every 5 bases under conditions of Poly PY • Poly PU • Poly PY reporter triplex formation (PU=Purine and PY=Pyrimidine) See Fig. 11B 4.

Step XI: Wash to remove unbound probe

Step XII: Initiate signal development by addition of the anti; DIG-Enzyme conjugate to the membrane bound reporter triplex. Up to 48 DIG molecules can be bound to the PNAS-reporter triplex.

Step XIII: Wash to remove unbound conjugate

Step XIV: Add a chromogenic substrate to the membrane resulting in an insoluble negatively charge precipitate being deposited on the membrane where target molecules and conjugate interacts to form a negatively charged colored precipitate that binds to the positively charged nylon membrane, like that shown in Fig. 11B 5.

Further signal amplification can be provided by use of, a self-complexing, biotinylated or other label substituted affinity molecule, signal amplification probe, resulting in a target localized density of biotin molecules to be next hybridized with the streptavidin-or other affinity pair-enzyme conjugate.

The last step in target detection requires the addition of a chromogenic substrate that will react with the enzymes, alkaline phosphatase, horseradish peroxidase or any similarly functional enzyme known to those skilled in the art. Presence of the Target DNA on the probe strip will be indicated by the development of an insoluble colored precipitate in the sample dot and positive control dot and no colored precipitate in the negative control dot. Signal development is well described in the literature and known by those skilled in the art. Exact experimental details must be empirically determined.

The preferred embodiment for the tube assay will possess 3 levels of specificity (1, TFO hybridization and triplex formation, 2, hybridization of capture probe, 3, hybridization of reporter probe). The gel assay would have the same levels with one additional level for target sizing (total four) by use of the restriction endonucleases..

An embodiment of the DNA TPA reverse dot blot format is presented in Figures 11A to 11B. The numerical designations represent the following:

1, the primary capture probe consisting of two regions 3, the 3' poly T tail (100-200Ts) and, 2, the 5' end capture probe.

2, the capture probe complementary to the PNAS/ 5' Tail structure

3, the poly dT (100-200 mer)

4, the triplex forming oligonucleotide (TFO)

5, the reporter region of the PNAS/Tails structure

6, the PNAS

7, the capture regions of the PNAS/Tail structure

8, the PNAS/5' Tails structure

9, the positively charged nylon membrane

10, the reporter probe composed of two sections, 19, the region complementary to the reporter tail of the PNAS/tail structure and 11

11, the label region, shown here as the 3' Poly dA (5'-3') region, the core for reporter triplex mediated signal amplification

12, normal hydrogen bonding

13, the poly dT 40 mer signal amplification probes

14, the poly dT (3'-5') 40 mer probe that forms a reporter duplex with the Poly dA region (antiparallel orientation binding)

15, the poly dT (5'-3') 40 mer probe that forms the reporter triplex reporter with the duplex region (parallel orientation binding)

16, Label, for example, biotin

17, the reporter triplex Poly dT (5'-3') • Poly dA (5'-3') poly dT (5'-3')

18, Hoogsteen's Bonding, weak hydrogen type bonding

19, Reporter probe region that binds to the PNAS/tail reporter region

DNA RFTA: Reverse Dot Blot Format

An embodiment of a DNA RFTA reverse dot blot assay format is presented in Figures 12A to 12B. The methods for this assay comprise the following steps. The example is directed to genomic DNA, though any nucleic acid can be used in the method with appropriate modifications.

Step I: DNA is isolated by any method known by those skilled in the art for genomic DNA isolation.

Step II: Restriction of the sample nucleic acid, for example, genomic DNA, releasing the dsDNA target.

Step III: Denature the dsDNA target with NaOH (alkali) or heat or other method dissociating the Watson (w) and Crick (c) strands.

Step IV: Primary probe hybridization. See Fig. 12A (1) The ssDNA segments are hybridized with a primary probe 2 that binds to only one part of the target creating a duplex structure with ss regions at both 5' ends. These ss 5' ends can function as either a capture region or a reporter region, in that either ss region can function as the reporter region or as the capture region. (First Level of Specificity) See Fig. 12A 1)

In Fig. 12A 1), the PDTP structure is shown. The capture region is generated by the 5' tail (primary probe 5' overlap) adjacent to the duplex partial target/primary probe moiety. The reporter region is generated by the 5' tail (target 5' overlap) on the opposite end of the duplex

partial target/primary probe moiety called the double stranded partial target probe with 2X 5' tails called PDTP/5' TAILS structure.

Step V: Production of nylon membrane with capture probes attached by T (thymidine) tailing and formation of a covalent crosslink between the Poly T links and the nylon membrane by UV 254 nm radiation was as described previously. See Fig 12A 2).

Step VI: Hybridize the PDTP complex to the capture probe affixed to the nylon membrane. (Second Level of Specificity) See Fig, 12A 3)

Step VII: Wash to remove non-specific nucleic acids.

Step VIII: Hybridize a reporter probe to the bound, PDTP Complex. The 5' region of the reporter probe of various length has a sequence complementary to the reporter region on the PDTP Complex. The 3' region of the reporter probe Poly dA also is of variable length; however, the poly dA region in this embodiment is preferably 120 nucleotides in length. (Third Level of Specificity) See Fig. 12A 4)

Step IX: Wash to remove unbound reporter probe.

Step X: Hybridize the 3' poly dA reporter region with a poly dT (40 mer, 3'-5' polarity, conjugated with Digoxygenin (DIG) at least every 5 bases under conditions of duplex formation. If the signal presented is insufficient it can be further amplified by placing another Poly dT probe (40 mer, 5'-3' polarity), conjugated with DIG at least every 5 bases under conditions of triplex formation. Up to 96 DIG molecules may be attached to a single Poly dA (120 mer) stretch and a single target.) See Fig. 12B 5.

Step XI: Wash to remove unbound signal amplification probes.

Step XII: Initiate signal development by addition of the anti; DIG-Enzyme conjugate; each Poly dT probe will attach 8 DIG molecules to the target (PDTP) Complex.

Step XIII: Wash to remove unbound conjugate.

Step XIV: Add a chromogenic substrate to the membrane resulting in an insoluble precipitate being deposited on the membrane where target molecules and conjugate interact to form a negatively charged colored precipitate that binds to the positively charged nylon membrane like that shown in Fig. 11B 5).

The preferred embodiment of DNA RFTA, Reverse Dot Blot Format has three levels of specificity (1, primary probe hybridization, 2, capture probe hybridization, 3, reporter probe hybridization).

Presence of the Target DNA on the probe strip will be indicated by the development of an insoluble colored precipitate in the sample dot, positive control dot, and no color in the

negative control dot. Signal development is well described in the literature and known by those skilled in the art, and must be empirically determined.

An embodiment of a DNA RFTA reverse dot blot format is presented in Figures 12A to 12B which numerical designations represent:

1, the target varying in 4 bases (A,T,G,C) or 2 bases (A,G or T,C)

2, the primary probe

3, the membrane bound capture probe with a 3' poly T end and a 5' end complementary to the capture region of the PDTP

4, the reporter region binding the complementary reporter probe at its 5' end and attaching the 5' end to the PDTP complex

5, the nylon membrane, usually additionally positive charged

6, the poly dT (3'end) tail section of the capture probe

7, the reporter probe with 2 sections, 1, 5' complementary to the reporter region and 2, the 3' poly dA signal amplification core structure

8, the section of the reporter probe that is complementary to the reporter region.

9, the poly dA, signal amplification core structure

10, Hoogsteen's Bonding, weak hydrogen bonding

11, normal hydrogen bonding between nucleic acid duplexes, and DNA and synthetic DNA ligands such as polyamide hairpins

12, the poly dT signal amplification probes pair 40 mer (3'-5' and 5'-3') conjugated with biotin or other label

13, the poly dT (3'-5') probe that hybridizes, antiparallel to the poly A region of the PNAS and PDTP conjugated with biotin forming the reporter duplex

14, the poly dT (5'-3') probe that hybridizes, parallel to the poly dA region of the dA/dT duplex conjugated with biotin or other label forming the reporter triplex

15, biotin, is the label used

16, the Partial Duplex Target Probe complex (PDTP)

17, the reporter triplex poly dT (5'-3') • poly dA (5'-3') • poly dT (3'-5')

18, the capture region of the PDTP

RNA TPA: Reverse Dot Blot Format

Direct RNA analysis has been difficult to achieve primarily due to the fact that PCR TAQ polymerase does not recognize nucleotides that do not contain a deoxyribose sugar. Currently, analysis of RNA targets is an indirect format requiring that a DNA copy (cDNA) of the RNA

target be produced; this is referred to as the Reverse Transcriptase step (RT) and the cDNA is then subjected to direct PCR analysis.

The RNA-TPA process offers direct RNA analysis with the ability to screen milligram quantities of RNA for select targets, assuring high sensitivity, with multiple specificity levels built into the system, assuring high specificity.

Direct RNA analysis will be instrumental in improving RNA virus detection (HIV, Hepatitis B, Hepatitis C), molecular metastasis and cancer detection, and any disease or hereditary gene that can be followed by analysis of a gene specific mRNA produced.

An embodiment of a RNA TPA Reverse Dot Blot assay format is presented in Figures 13A and 13B. The methods for this assay comprise the following steps.

Step I: Isolation of RNA, in this example, messenger RNA, by any method known by those skilled in the art. Care must be taken to protect the mRNA molecules isolated from exposure to environmental ribonucleases that will degrade the mRNA, resulting in target loss. See Fig. 13A 1.

Step II: Production of a capture probe affixed nylon membrane as described previously. See Fig. 13A 2

Step III: Hybridization of a second complimentary capture probe to the first capture probe which attached to the membrane, to form a complete dsDNA capture probe. A duplex hairpin may also be used offering 1 less level of specificity.

The second step in mRNA TPA is production of a nylon membrane containing multiple probes (previously discussed) with appropriate positive and negative controls. The capture probe on the membrane is a ssDNA with a 3' Poly (dT) region covalently bound to the membrane by UV (254nm) irradiation as previously discussed, and the 5' end (capture probe) that consists of a ssDNA region that will partly confer sequence specificity for binding of the unique mRNA target sequence, via interaction with the 5' capture region of the PNAS/(5'tails) structure.

The single capture probe attached to the membrane is hybridized with a complementary probe to the 5' end of the capture probe that will result in a duplex capture probe with complete sequence specificity for target binding and triplex formation. This is the first level of specificity of the assay. The target region is RNA (a single stranded polypurine or polypyrimidine segment) and the capture probe is a double stranded DNA molecule attached to the nylon membrane that evidences itself as a polypurine and polypyrimidine rich duplex region. Hybridization conditions are known to those skilled in the art and have previously been discussed. (First Level of Specificity) See Fig. 13A 3)

Step IV: Wash to remove unbound capture probe

Step V: Hybridize the mRNA sample with the dsDNA membrane bound capture probes in order to generate the triplex PNAS. For example, the (w) strand of the capture duplex would be a polypurine region, the (c) strand of the capture duplex would be a polypyrimidine region, and the mRNA subspecies specific target is a polypyrimidine segment to allow formation of the triplex Poly Py • Poly Pu• Poly Py. (Second Level of Specificity) See Fig. 13A 4)

The triplex protected mRNA target consists of an mRNA with a free-floating 5' capped end and 3' Poly A region of 200 to 250 adenine bases. The 3' Poly A end will become important later in signal amplification for target detection. The 3' polyA end may be a substrate for ss exonuclease degredation, and if it is not protected, would degraded and leave a blunt end on the PNAS. Another strategy is to hybridize another reporter probe also posessing a poly dA region to another site between the triplex (PNAS) and the 5' conserved end of the mRNA species specific target.

Step VI: Wash to remove non-specific mRNA (may be optional), due to enzyme degredation of this element that follows.

Step VII: hybridize with a reporter probe. See Fig. 13B 5)

Step VIII: wash (optional step)

Step IX: Treat the membrane and free-floating mRNAs with an enzyme such as EXO III (exodeoxyribonuclease) to degrade all capture probes not protected by the triplex PNAS, and a ss exonuclease to remove the poly dA region of the entire messenger RNA population. See Fig. 13B 6).

The hybridized membrane can be treated with EXO III to remove all dsDNA capture probes that have not been protected by binding of the RNA target. Remember that in RNA TPA, the target protects the probe and the EXO III functions to destroy non-protected dsDNA capture probe, and the poly dA signal initiating element for all mRNA molecules (target and non-specific RNA that might interfere and deliver a false result in this embodiment would be resolved by the destruction of the poly A region of the entire mRNA population. This step increases the overall specificity of the assay and will be considered the third level of specificity. (Third Level of Specificity)

Step X: Wash to remove all enzymes

Step XI: Hybridize the nylon membrane with Poly dT (5'-3' and 3'-5') signal amplification reporter probes in molar excess concentration over targets present. See Fig. 13B 7).

Signal amplification can now be achieved by hybridizing a poly T probe of finite variable length, conjugated with DIG at every nth base to the target complex. Hybridization conditions

and periodicity of DIG conjugation are known to those skilled in the art and have been previously discussed. Furthermore, the probe length can be decided on a cost of synthesis basis (40 bases in length can be manufactured, cost efficiently). Furthermore, since the A-T hydrogen bonding is weaker than the G-C pair, a 40 mer oligo an optimal size, length vs. stability of the reporter duplex and the reporter triplex.

The poly dT 40 mer (pair) (3'-5') and (5'-3') hybridization will allow up to 12 Poly dT probes (total) to bind to the mRNA 3' Poly A end (250 mer). Since each Poly dT probe has eight DIG bound per the 40 mer Poly dT probe resulting in up to 96 opportunities for binding of the anti; DIG-Enzyme conjugate.

Step XII: Wash to remove unbound signal amplification probes (pairs)

Step XIII: Initiate signal development by addition of the anti DIG-Enzyme conjugate. One molecule of DIG binds one molecule of conjugate and each poly dT probe (pair) has 8 molecules of DIG and subsequently 8 molecules of conjugate bound to it.

Step XIV: Wash to remove unbound conjugate.

Step XV: Addition of a chromogenic substrate to the membrane resulting in an insoluble colored precipitate being deposited by charge on the membrane where target molecules and conjugate are located.

Presence of the target mRNA on the probe strip will be indicated by the development of an insoluble colored precipitate in the sample dot, positive control dot and no color in the negative control dot.

Signal development is well described in the literature and known by those skilled in the art and must be empirically determined.

The preferred embodiment of this format will possess 3 levels of specificity (1, hybridization of the complementary capture probe DNA forming a dsDNA capture probe bound to the nylon membrane, 2, binding of the mRNA target to the duplex probe forming a triplex PNAS, 3, EXO III and ss exonuclease degradation of unprotected DNA dsDNA probes and ss mRNA targets, respectively..

Another embodiment may involve use of a dsDNA closed hairpin to capture and form a PNAS with a unique mRNA polypyrimidine segment.

The numerical designations in Figures 13A to 13B are:

1, the mRNA specific subspecies

2, the target (polypyrimidine region)

3 the specific 3' non-coding mRNA region

4, the poly A (~250 mer) 3' end of the mRNA molecule

5, the 3' poly dT (T-tail) of the primary capture probe

6, the positively charged nylon membrane

7, the capture region with sequence specificity for the mRNA polypyrimidine target

8, the primary capture probe with two sections, 7, the capture (W) 5' end (polypurine) and, 5, the 3' T-tail (100-200 mer)

9, the secondary capture probe, in this example, characterized as a polypurine rich region (W) strand forming a duplex with the polypyrimidine (C) strand 7

10, the 5' cap on mRNA molecules rendering the end exonuclease resistant.

11, normal hydrogen bonding forming the duplex capture probe.

12, Hoogsteen Bonding weak hydrogen bonding

14, the poly dT signal amplification probes (pair)

15, the poly dT (3'-5') 40 mer probe binding antiparallel to the poly dA region forming a reporter duplex

16, the poly dT (5'-3') 40 mer probe binding parallel, to the poly dA region forming a reporter triplex

17, label, for example, biotin is the label used

19, the reporter triplex (Poly dT (5'-3') • Poly dA (5'-3') • Poly dT (3'-5')

20, the subspecies specific 5' encoding region of mRNA

21, poly A region of reporter probe, the probe may have a 5' or 3' cap

RNA RFTA Reverse Dot Blot

The RNA RFTA process provides a number of specificity levels designed into the process, resulting in high test specificity levels.

Direct RNA analysis will be instrumental in improving RNA virus detection (HIV, Hepatitis B, Hepatitis C), molecular metastasis and cancer detection, and any disease or hereditary gene that can be followed by analysis of the gene specific mRNA produced.

The RNA RFTA reverse dot blot format is described in Figure 14A to 14B:

Step I: Isolation of RNA, specifically mRNA, by any method known by those skilled in the art. See Fig. 14A 1.

Step II: Production of the capture probe affixed nylon membrane by attachment of specific capture probes that have a 5' T-tail of 100-200 Ts as described above and shown in Fig. 14A 2.

Step III: Hybridize the membrane bound capture probes with the total mRNA sample. (First Level of Specificity). See Fig. 14A 3.

The advantage of RFTA over TPA is that any 4 base variation site can be considered as part of the duplex containing target. In TPA, the target preferably comprises a 2 base variation (polypurines, and polypyrimidines).

Step IV: Wash to remove non-specific mRNA (this step is optional).

Step V: Hybridize with a reporter probe which has specificity off binding to the 5' coding end of the mRNA molecule(target mRNA subspecies specific region) to a region between the target and the 5' capped mRNA end. (Second Level of Specificity) See Fig. 14A 4.

Step VI: Wash to remove unbound reporter probe (this step may be optional).

Step VII: treat with ss (3'→5') RNA exonuclease. This proffers the third level of specificity of the assay by destroying unbound reporter probes and all naturally occurring 3' poly A regions on the total mRNA population. See Fig. 14B 5.

Step VIII: wash to remove enzyme

Step IX: Hybridize the nylon membrane with labeled poly dT, 40 mer signal amplification probes Poly dT, (3'-5') and Poly dT (5'-3'). See Fig. 14B 6).

These Poly dT probes are used for signal amplification to detect the presence of the PDTP complex in the sample. The Poly A (or dA) (5'-3') first interacts with the Poly dT (3'-5') in an antiparallel fashion, forming the duplex Poly A • Poly dT (3'-5'). Considering the length of the Poly A (~250 mer) region, up to the 6 Poly dT (3'-5') probes can be accommodated (~240 mer). This would result in 48 DIG molecules to be stably added to the PDTP complex. If further signal amplification is necessary, the duplex can be reacted with the other Poly dT pair, Poly dT (5'-3'), having a parallel orientation with the (w) Poly A and under conditions favoring triplex formation known to those skilled in the art, provide up to 96 DIG molecules to be stably added to the PDTP complex for attachment of the anti; DIG-Enzyme conjugate.

Step X: Wash to remove unbound signal amplification reporter to probes.

Step XI: Initiate signal development by addition of the anti; DIG-Enzyme conjugate. There is a one to one ratio of DIG to enzyme molecule and up to 2 logs of increased sensitivity by use of the reporter triplex. Each enzyme would process the chromogenic substrate molecules with a turnover of 3,000 to 5,000 substrate molecules to a negatively charged colored precipitate.

Step XII: wash to remove unbound conjugate.

Step XIII: Addition of a chromogenic substrate to the membrane resulting in an insoluble precipitate being deposited on the membrane where target molecules and conjugate interact.

The final step in target detection requires the addition of a chromogenic substrate that will react with the enzymes, alkaline phosphatase, horseradish peroxidase or any functionally similar enzyme known to those skilled in the art. Presence of the target on the probe strip will be indicated by the development of an insoluble colored precipitate in the sample dot, positive control, dot, and no color in the negative control dot. Signal development is well described in the literature and known by those skilled in the art.

Numerical designations in Figures 14A and 14B, presented in the RNA RFTA assay are:

1, the mRNA subspecies specific molecule

2, the target region of a specific mRNA subspecies

3, the mRNA specific 3' non-coding region present in all mRNAs

4, the poly A (~250 mer) 3' end of all mRNA molecules

5, the 3' poly dT tail of the primary capture probe

6, the positively charged nylon membrane

7, the sequence specific mRNA target-capture region of the capture probe

8, the primary capture probe consisting of two sections, 5, the 3' poly dT tail and, 7, the 5' capture region.

9, the Partial Duplex Target Probe complex

10, the cap on the 5' end of all mRNA rendering it 5' exonuclease resistant.

11, normal hydrogen bonding, connecting the capture region and the mRNA subspecies specific target

12, the labeled poly dT signal amplification probes

13, the poly dT (3'-5') 40 mer signal amplification probe that forms a duplex reporter with the 3' Poly A region of the mRNA molecule, binding in an antiparallel fashion

14, the poly dT (5'-3') 40 mer signal amplification probe that forms a triplex reporter with the (5'-3') Poly A • (3'-5') Poly dT duplex binding in antiparallel fashion.

15, The label, in this example, biotin is the label used

16, the reporter triplex poly dT (5'-3') • Poly dA (5'-3') • Poly dT (3'-5')

17, the mRNA subspecies specific 5' encoding region

18, reporter probe

19, poly dA region (signal amplification core region)

The Multiple Triplex Reporter Forming Self-Complexing Probes (MTRF probes)

The MTRF probes of the present invention provide efficient and multiple log signal amplification for detection of the low copy number target/probe complexes (PNAS and PDTP). One embodiment of a novel method for signal amplification, namely the affinity molecule conjugated Poly dT probe (pairs), has previously been discussed. In some assays, more label may be necessary for more sensitive target detection (Duplex and Triplex Reporter and MTRF).

To increase the number of label molecules bound to the target, the present invention comprises a self-complexing reporter probe group that allows large numbers of any label to be bound to the PNAS and PDTP complexes. Figure 15-I represents the Poly A region of the mRNA molecule or the Poly dA region of the reporter probe, to which is hybridized signal amplification probes (Poly dT (5'-3' and 3'-5'). In this embodiment at least 12 Poly dT probes (40 mer) can form a reporter triplex making 96 label molecules (8 affinity molecules on each of the poly dT probes) available for signal amplification.

The initiator probes may have a Poly dT region complementary to the Poly A or Poly dA reporter regions on the PNAS and PDTP, and have a region for initiation of the MTRF cascade. These probes provide the signal amplification for the Poly A section of the TPA reporter and the Poly A of the RFTA primary probe reporter and each has two sections complementary to the reporter region of the PNAS and PDTP complex which is the Poly A mRNA region and the Poly dA DNA region. Section, 2, must be polypyrimidine and must form a Poly Py • Poly Pu • Poly Py reporter triplex with probes MTRF-A and MTRF-B.

The MTRF Initiator probe is the starting probe on which the rest of the MTRF structure is formed. The initiator probe can bind to the PNAS, PDTP, or other structures formed in association with the target sequence. The MTRF Initiator probe can be bound to the target sequence structures, such as PNAS and PDTP structures, by a 4 base varying complementary region as well as to polypyrimidine or polypurine regions. These probes, provide the signal amplification for the Poly A or Poly dA section of the TPA reporter and the Poly A or Poly dA of the RFTA reporter probe, each having the3' poly A or dA end in the mRNA and DNA assays, respectively. Each also has sections complementary to the reporter region of the PNAS and PDTP complex which is the 3' Poly A mRNA region and the 3' Poly dA DNA region for DNA assays. Each must have a 5' polypyrimidine end, which forms a Poly Py • Poly Pu • Poly Py reporter triplex with probes MTRF-A and MTRF-B.

Figures 15 and 16 present the MTRF probes and their anchor region (Poly dT-also poly dC) or a 4 base varying anchor section of the MTRF initiators) with complementarity to a region on the 5' end of the PNAS or PDTP structure.

In the embodiment where a poly dA or poly A region is used for MTRF initiation, the MTRF core signal amplification sequence may be modified from the poly dT • poly dA • poly dT triplex to a poly C • poly G • poly C triplex. This would prevent exchange of initiator and triplex reporter sequences (the poly dT probes).

Figure 15-II represents the level of organization of the MTRF probes in this embodiment (the "nucleus") as well as the initiator strategies to attach the self-complexing MTRF probes to the PNAS and PDTP structures. This includes possessing a reporter probe with section A or B and MTRF initiator probes with a complementary sequence A' or B' that activates the MTRF cascade.

MTRF Initiator

The MTRF initiator probes bind anti-parallel to the reporter region on the PNAS and PDTP forming a duplex and having a 3' end region that binds to a 2 base (A,T or G,C) polyrich region or to a 4 base varying region, both of which possess a 5' end region that is polypyrimidine that initiates the MTRF probes cascade.

It is important to note that the association of the MTRF Initiator probes with the poly A may prove problematic due to the similar presence of poly dA regions in the other MTRF amplification complex probes. Thus, one embodiment of the present invention contemplates use of another 4 base varying region for anchorage of the MTRF Initiator Probe, thereby limiting exchange reactions between the poly A mRNA region, the poly dA regions of the MTRF probes, and the poly dT signal amplification probes. The initiator probes for all RNA assays, including mRNA, rRNA or tRNA, or DNA assays may have any sequence complementary to the target itself, or to an oligonucleotide with a sequence that is complementary to the target itself (e.g., a reporter probe).

MTRF A and MTRF-B Probes:

Both probes interact with a region on the initiator and each end has a unique and different polypyrimidine or polypurine sequence. Both probes interact with the A' and B' different end regions of different and complementary initiator probes and each has a unique polypyrimidine sequence that initiates the MTRF cascade.

All MTRF probes anchor to the core, poly A or dA, or other sequences and have different end region sequences in order to form and close the box around the MTRF Initiator 5' end polypyrimidine sequence.

As demonstrated in Figure 16, two MTRF probes bind to the polypyrimidine region (Section 2) by formation of the triplex (Poly Py • Poly Pu • Poly Py). Probes MTRF-A and MTRF-B have the following structure in one embodiment:

	region 1	region 2	region 3
MTRF-A	Poly Pu (18 mer)	Poly dA (400 mer)	Poly Pu (18 mer)
MTRF-B	Poly Py (18 mer)	Poly dA (400 mer)	Poly Py (18 mer)

MTRF-A: 3' Poly PU-signal core-poly dA-Poly PU 5'

MTRF-B: 3' Poly PYR-signal core poly dA-Poly PYR 5'

MTRF-C and MTRF-D Probes:

These probes connect the various quatroplex probe complexes as represented in Figure 15-II, and have the following structure:

MTRF-C: 3' Poly PU-signal core poly dA-Poly PU 5'

MTRF-D: 3' Poly PYR-signal core poly dA-Poly PYR 5'

Another embodiment of the present invention comprises probes characterized by:

MTRF-A:

5' Poly PYR-signal core with a reverse in polarity-Poly PYR 5'

MTRF-B:

3' Poly PU-signal core with a reverse in polarity-Poly PU 3'

MTRF-C:

5' Poly PYR-signal core with a reverse in polarity-Poly PYR 5'

MTRF-D:

3' Poly PU-signal core with a reverse in polarity-Poly PU 3'

These reversed polarity probes are presented and discussed later in this document. The overall MTRF complex structure should not be limited to any one configuration, but to include all possible configurations known by those skilled in the art resulting in numerous different embodiments.

Though the mRNA signal generating 3' end is referred to as approximately a 250 base region, the present invention is not limited to embodiments consisting of only this number of

bases or any polyrich region, but instead, is capable of posessing any size or any base sequence at the 3' end that forms a triplex with the corresponding labeled signal amplification probes. Furthermore, the poly dA reporter probe is referred to as having a 120 mer Poly dA region; however, it must be understood that the length of this region (Poly dA) is simply a function of cost efficiency of the polynucleotide synthesis. Current oligonucleotide synthesis technology can cost efficiently provide a Poly dA and Poly A region of 50 to 100 bases. The 120 mer Poly dA will remain throughout the test, and the DIG calculations will stand; however, these assays can accommodate as long a Poly dA region that can be produced with cost efficiency.

Numerical designations for embodiments represented in Figures 15 and 16 representing the MTRF self-complexing probes for enhanced signal amplification are:

1a, the reporter probe in the DNA assay with two sections, 1, a 3' capped or 5' Poly dA or 4 base varying region and, 2, a 5' end region complementary to the reporter region on the PNAS and PDTP structures.

1b, the reporter probe in the mRNA assay with two sections, 1, a 3' cap Poly dA or 4 base varying region and, 2, a 5' end region complementary to the reporter region on the PNAS and PDTP structures.

2a, the MTRF initiator probe anchor region, which has at its 3' end a poly dT sequence, binding antiparallel to the poly dA region of the DNA poly dA reporter region or the poly A mRNA reporter region on the PNAS and PDTP.

2b, the MTRF Initiator probe possesses two sections, 1, a 3' anchor region, poly dT binding antiparallel to the Poly A (mRNA) 5'-3' reporter probe section, and, 2, a 5' end polypyrimidine region that binds to a reporter probe forming a triplex with MTRF-A and MTRF-B in order to initiate the MTRF cascade of probes.

2c, the polypyrimidine MTRF initiation sequence forming a triplex with MTRF A and MTRF D.

3a, the MTRF initiator probe anchor region, which has at its 3' end a 4 base varying sequence, binding antiparallel to the 4 base varying reporter region on the PNAS or PDTP structures for both DNA and RNA assays.

3b, the MTRF initiator probe posesses two sections, 1, a 3' 4 base varying end which is complementary to a region on the PNAS or PDTP reporter region in DNA or RNA assays, and, 2, a 5' end polypyrimidine region forming a triplex with MTRF-A and MTRF-B in order to initiate the MTRF cascade of probes.

3c, the polypyrimidine MTRF initiation sequence forming a triplex with MTRF A and MTRF D.

4a, the polypurine sections of the reporter probe region or a four base varying sequence on the 3' end of the reporter probe.

4b, the MTRF Initiator anchor region complementary to the reporter region

5, the Duplex Reporter Formation

6, the Triplex Reporter Formation

7a, the Multiple Triplex Reporter Formation, MTRF A, probe composed of 3 sections, 1, the 3' end a polypurine region, 2, a central poly dA region, and 3, a 5' end polypurine region.

7b, the Multiple Triplex Reporter Formation, MTRF C, probe composed of 3 sections, 1, the 3' end a polypurine region, 2, a central poly dA region, and 3, a 5' end polypurine region.

8a, the 5' end polypurine region of MTRF A (with a unique sequence)

8b, the 3' end polypurine region of MTRF C (with a unique sequence)

9, the poly dA region of the MTRF which acts as a core for signal amplification utilizing triplex reporter formation.

10a, the 3' end polypurine region of MTRF A (with a unique sequence)

10b, the 5'end polypurine region of MTRF C (with a unique sequence)

11a, the MTRF B probe with three sections 1, the 5' end a polypyrimidine region, 2, a central Poly dA region, and, 3, a 3' end polypyrimidine region.

11b, the MTRF D probe with three sections 1, the 5' end a polypyrimidine region, 2, a central Poly dA region, and, 3, a 3' end polypyrimidine region.

12a, the 5' end of the MTRF B polypyrimidine region (with a unique sequence)

12b, the 3' end of the MTRF D polypyrimidine region (with a unique sequence)

13a, the 3', end of the MTRF B polypyrimidine region (with a unique sequence)

13b, the 5' end of the MTRF D polypyrimidine region (with a unique sequence)

14, the MTRF-initiator anchor region which is a Poly dT or 4 base varying region that binds antiparallel to the reporter region on the reporter probe attached to the PNAS and the PDTP structure.

15a, the MTRF nucleus connector probe with 3'x 3' reversed polarity in the middle across a 12 carbon spacer (poly py - carbon spacer - poly py) attaching probes MTRF C and D (pu-pyr duplex) in one "nucleus" to MTRF probes A and B (pu-pyr duplex) in a second "nucleus" via triplex formation or vice versa depending on the direction of MTRF complex extension; each section has a different sequence that is identical to the polypyrimidine region sequence in the local triplex formation area, but will bind with opposite orientation, namely parallel to the polypurine regions in each duplex

15b, the MTRF "nucleus" connector probe with 3' x 3' reversed polarity in the middle across a 12 carbon spacer (poly py - carbon spacer - poly py) attaching probes MTRF A and B (pu-pyr duplex) in one "nucleus" to MTRF probes C and D (pu-pyr duplex) in a second "nucleus" via triplex formation or vice versa depending on the direction of MTRF complex extension; each section has a different sequence that is identical to the polypyrimidine region sequence in the local triplex formation area, but will bind with opposite orientation, namely parallel to the polypurine regions in each duplex

16a, the MTRF "nucleus" connector probe with 5'x 5' reversed polarity in the middle across a 12 carbon spacer (poly py - carbon spacer - poly py) attaching probes MTRF C and B (pu-pyr duplex) in one "nucleus" to MTRF probes A and D (pu-pyr duplex) in a second "nucleus" via triplex formation or vice versa depending on the direction of MTRF complex extension; each section has a different sequence that is identical to the polypyrimidine region sequence in the local triplex formation area, but will bind with opposite orientation, namely parallel to the polypurine regions in each duplex

16b, the MTRF "nucleus" connector probe with 5'x 5' reversed polarity in the middle across a 12 carbon spacer (poly py - carbon spacer - poly py) attaching probes MTRF A and D (pu-pyr duplex) in one "nucleus" to MTRF probes C and B (pu-pyr duplex) in a second "nucleus" via triplex formation or vice versa depending on the direction of MTRF complex extension; each section has a different sequence that is identical to the polypyrimidine region sequence in the local triplex formation area, 'but will bind with opposite orientation, namely parallel to the polypurine regions in each duplex

17, the poly dT signal amplification probes, poly dT (3'-5' orientation) and (5'-3'orientation) binding to the poly dA core region forming respectively a reporter duplex and a reporter triplex, while stabilizing the hydrophobic center of the MTRF Nucleus.

18, the poly dT (40 mer) 3'-5' probe forming a duplex reporter with poly dA (5'-3')

19, the poly dT (40 mer) 5'-3' probe forming a triplex reporter with the duplex poly dA (5'-3') poly dT (T-5').

20, the MTRF "nucleus" structure

The present invention is directed to compositions and methods for molecular biological techniques. More particularly, the present invention can be used for signal amplifications procedures for nucleotide sequence analysis.

In one embodiment of the present invention, MTRF (Multiple Triplex Reporter forming) self-complexing probes are provided for analysis processes that do not include a restriction step,

enzymatic or nonenzymatic. In summary, a preferred embodiment comprises one section of the initiator probe, MTRF 1, bound in a complementary manner to a native double strand DNA target in vitro without any need for restriction. Ideally, the DNA is first purified. The probe hydbridizes and results in local triplex formation which can be stabilized by certain experimental conditions, including, but not limited to, salts, pH condition or temperature. Additionally, photopsoralens can be added to cross-link the triplex strands. If an mRNA sample is to be analyzed in another embodiment, a duplex structure is formed with the initiator probe. This duplex structure is more stable than a triplex structure and thus requires fewer conditions to maintain the stability.

The next step involves the addition of other MTRF probes. In one embodiment, eight other probes are added, shown in Figure 18 as MTRF Probes 2-9. The self-complexing probe complex formed will undergo a primary nucleus formation as well as other complex additions to the nucleus. This nucleus formation is referred to as "the MTRF-DNA complex" or "the MTRF-RNA complex". The probes of the present invention may be labeled with any labels known to those skilled in the art. Such labels include, but are not limited to, labels such as chemical; enzymatic; radioactive; biotin; binding pairs such as biotin/streptavidin; antibodies/antigens; immune fragments; and chemiluminescence and bioluminescence. Such labels are detected by detection systems known to those skilled in the art.

A preferred method of the present invention comprises MTRF probes that are labeled and detected with the binding pair biotin/streptavidin. For example, MTRF complexes' polydA regions interact with biotinylated polydT signal generation probes and produce complex formations. The biotin molecules are attached to poly dT probes which bind to the intial probes complexed with the target DNA or RNA sequences. Target detection is accomplished by adding a straptavidin/Aquorin conjugate to the bound biotin probes. In this example, target is detected by the bioluminescence of the aquorin molecule in the presence of calcium in a luminometer.

This method of detection can be used when multiple target sequences are detected, such as in a general multiplex screening procedure. Each target sequence is bound by a specific probe, followed by addition of the MTRF probes and them the poly dT probes, labeled with biotin and then reacted with the aquorin/streptavidin conjugate. The presence of multiple targets would be detected in a luminometer. Further differentiation of individual specific targets is accomplished in additional tests.

The MTRF methods and compositions can be used to detect very low copy number of target sequences. The present invention can be configured in two or three specificity steps to detect low copy number target sequences using a high signal amplification reaction for detection.

A preferred method of the present invention comprises three steps. The first step comprises binding of a capture probe to the nucleic acid containing the target sequence. A second step comprises binding of a reporter/initiator probe to a sequence different from that bound by the capture probe. The third step comprises binding of labeled probes to the complex formed by the initiator probe and other MTRF probes, and detection of the label. Additional steps, such as use of a triplex forming reporter/initiator probe, and enzyme treatments to reduce the nonspecific nucleic acids that are present, and other molecular biological treatments known to those in the art, are contemplated by the present invention.

Preferred embodiments of methods for the detection of DNA, MTRF-DNA, are herein described. One embodiment of a MTRF-DNA method identifies single-stranded DNA target sequences and comprises duplex forming capture robes and duplex forming reporter probes that each bind to different sequences of the ssDNA. A second embodiment of MTRF-DNA comprises use of a primary capture probe that is complementary to a region of a target sequence, and forms a duplex with the ssDNA when bound. "This embodiment also comprises a second duplex forming probe, sspolypyrimidine in nature, that is complementary and binds antiparallel to the ssDNA target, which would be the polypurine (W,strand) and a third polypyrimidine containing Triplex Forming Oligonucleotide (TFO) acting [as a reporter/initiator probe] binding parallel to the(W) strand of the polyrich duplex also within the target region, initiating the MTRF reporter cascade.

A further embodiment identifies a dsDNA target (polypurine (W) and polypyrimidine (C) in nature. Presence of the two polyrich duplexes in the target region permits the parallel binding of the capture TFO and the polypyrimidine (TFO) region of MTRF1 to the W strand, also within the target region, initiating the MTRF reporter cascade.

An embodiment of a composition and method of the present invention is shown in Figure 17. The probes used in the present disclosure can be synthesized using known techniques. The sequences used in this embodiment are for illustration and are not to be limiting on the scope of the invention.

Figure 17 shows MTRF nucleus or primary structure. The numerical designations are as follows:

1, is the MTRF initiator probe. This probe comprises two oligonucleotides joined at their 3' ends, so that each end of the probe has a 5' polarity. This probe comprises 4 sections. "The 5' end is complementary to the target sequence, followed by a series of three 18 atom spacer molecules (equivalent to a 12 cytosine strand) followed by a polypyrimidine region of approximately 15 [preferably 8-25, would prefer 15-19] bases in length, and another

polypyrimidine region (also 15 bases) with a reversal of polarity" between the two polypyrimidine regions.

2, is MTRF 2 probe, comprising two oliogonucleotides joined at the 3' ends. These two oligonucleotide sequences may be joined so that the polarity of one is the reverse of the polarity of the second. For example, the 3' end of one oligonucleotide is joined to the 3' end of the other oligonucleotide. Each oligonucleotide comprises a 5' polypyrimidine sequence and a 3' poly dA sequence. The two oligonucleotides are joined at the 3' end by any method known to those skilled in the art.

3, is MTRF 3 probe, comprising two oliogonucleotides joined at the 5' ends. These two oligonucleotide sequences may be joined so that the polarity of one is the reverse of the polarity of the second. For example, the 5' end of one oligoucleotide is joined to the 5' end of the other oligonucleotide. Each oligonucleotide comprises a 3' polypurine sequence and a 5' poly dA sequence. This sequence provides the core structure for signal amplification. The two oligonucleotides are joined at the 5' end by any method known to those skilled in the art.

4, is MTRF 4 probe, comprising two oligonucleotides joined at the 3' ends. These two oligonucleotide sequences may be joined so that the polarity of one is the reverse of the polarity of the second. For example, the 3' end of one oligonucleotide is joined to the 3' end of the other oligonucleotide. Each oligonucleotide comprises a 5' polypyrimidine sequence and a 3' poly dA sequence. The two oligonucleotides are joined at the 3' end by any method known to those skilled in the art.

5, is MTRF 5 probe, comprising two oliogonucleotides joined at the 5' ends. These two oligonucleotide sequences may be joined so that the polarity of one is the reverse of the polarity of the second. For example, the 5' end of one oligoucleotide is joined to the 5' end of the other oligonucleotide. Each oligonucleotide comprises a 3' polypurine sequence and a 5' poly dA sequence. This sequence provides the core structure for signal amplification. The two oligonucleotides are joined at the 3' end by any method known to those skilled in the art.

6, is MTRF 6 probe comprising two oligonucleotides joined at the 3' ends. These two oligonucleotide sequences may be joined so that the polarity of one is the reverse of the polarity of the second. For example, the 3' end of one oligonucleotide is joined to the 3' end of the other oligonucleotide. Each oligonucleotide is a polypyrimidine sequence. The two oligonucleotides are joined at the 3' end by any method known to those skilled in the art. The polypyrimidine sequences of this probe are identical to the initiator probe's polypyrimidine sequence.

7, is MTRF 7 probe comprising two oligonucleotides joined at the 3' ends. These two oligonucleotide sequences may be joined so that the polarity of one is the reverse of the polarity

of the second. For example, the 3' end of one oligonucleotide is joined to the 3' end of the other oligonucleotide. Each oligonucleotide is a polypyrimidine sequence. The two oligonucleotides are joined at the 3' end by any method known to those skilled in the art.

8, is MTRF 8 probe comprising two oligonucleotides joined at the 3' ends. These two oligonucleotide sequences may be joined so that the polarity of one is the reverse of the polarity of the second. For example, the 3' end of one oligonucleotide is joined to the 3' end of the other oligonucleotide. Each oligonucleotide is a polypyrimidine sequence. The two oligonucleotides are joined at the 3' end by any method known to those skilled in the art.

9, is MTRF 9 probe comprising two oligonucleotides joined at the 3' ends. These two oligonucleotide sequences may be joined so that the polarity of one is the reverse of the polarity of the second. For example, the 3' end of one oligonucleotide is joined to the 3' end of the other oligonucleotide. Each oligonucleotide is a polypyrimidine sequence. The two oligonucleotides are joined at the 3' end by any method known to those skilled in the art.

10, is the poly dA region, preferably comprising 1000 nucleotides.

11, is the poly dA region, preferably comprising 250 nucleotides.

12, is a triplex reporter complex, comprising poly dT, poly dA, polydT

13, is a triplex reporter complex, comprising poly dT, poly dA, polydT

14, is the region of the MTRF 1 that is complementary to a specific sequence on the target/probe complexes.

15, is the poly dT labeled oligonucleotides, preferably comprising approximately 40 nucleotides and labeled with biotin.

16, is the spacer region, preferably three eighteen atom spacers.

Figure 18 is an embodiment of the present invention that illustrates the interaction of several of MTRF probe forming complexes. The numbering is the same as in Figure 17.

Figure 19 is an illustration of multiple interacting MTRF probe complexes. The complexes may be stabilized by methods known to those skilled in the art such a photopsoralen stabilization methods and others. The numbering is the same as Figure 17.

As an example of the use of Aquorin as a the detection method, Table 3 shows the use of the MTRF signal amplification methods using the binding pair of biotin and streptavidin/Aquorin. The numbers in Table are reflect the current requirements of Aquorin sensitivity, e.g., that the current lower limits of sensitivity of Aquorin is 300,000 Aquorin molecules. These numbers are presented as illustration only and the present invention comprises other detection systems with different sensitivities.

Table 3

Round of Formation	Number of MTRF Nuclei	Number of Biotin molecules attached to target	Minimal number of targets detected by Aquorin bioluminescence
1	1	1,000	300
2	4	4,000	60
3	8	8,000	23
4	12	12,000	12
5	16	16,000	7
6	20	20,000	5

One embodiment of the MTRF signal amplification methods and compositions with DNA comprise the following steps.

Step 1: DNA is isolated and can be subjected to restriction enzymes or physical shearing if necessary.

Step 2: The DNA is denatured using techniques known to those skilled in the art, such as with heat or alkaline conditions.

Step 3: Hybridize one strand of the DNA with an aminated (a primary amine NH2-) capture probe that binds antiparallel [complimentary] to the capture region in the target region, forming a duplex structure. The capture probe has a primary amine attached via a spacer region, preferably an 18 atom spacer. After hybridization, a single strand specific nuclease can be added to remove single stranded DNA.

Step 4: The MTRF initiator probe is added. This probe hybridizes to the target region via complementary sequence region of the probe, and binds in an antiparallel fashion to the target sequence. The other regions of the MTRF initiator probe are as previously described.

Step 5: Wash to remove unbound probes.

Step 6: Add the MTRF complex of probes, preferably MTRF 2-8, to generate the MTRF signal amplification system. The order of MTRF probe addition would be as follows for one embodiment:

1. interaction of the target/probe complex with the reporter initiator probe MTRF1 with incubator and wash.

2. add MTRF probes 2-5 to form individual "nuclei" for signal generation with incubation and wash.

3. add MTRF probes 6-9 to join individual "nuclei" forming a multi-level complex for signal generation with incubation and wash.

Step 7: Wash to remove unbound probes.

Step 8: An optional step of UV radiation if photopsoralen is incorporated in the probes, to stabilize the structures formed.

Step 9: Add the biotinylated poly dT signal generation probes. Preferably, these probes are approximately 40 nucleotides. These probes bind to the poly dA regions in the MTRF complex to form the duplex and triplex signal regions.

Step 10: Wash to remove unbound poly dT-biotin probes.

Step 11: Add Streptavidin/Aquorin conjugate in sufficient amount to bind to the biotin molecules.

Step 12: Wash to remove unbound conjugate.

Step 13: Add calcium to activate the aquorin light production and detect signal using a luminometer.

Numbers used in Figure 20 are as follows:

1, is the denatured DNA

2, is the polypurine target region

3, is the duplex forming primary capture probe characterized as having a primary amine or any other biochemical hook attached to the 3' or 5' end, preferably using a spacer molecule.

4, is the spacer molecule.

5, a primary amine is the biochemical hook and binds to N-oxysuccinimide (NOS) coated plates, etc.

6, is the MTRF initiator probe.

7, is the 5' end of the MTRF probe that binds to the 4 base varying sequence of the target sequence. All four bases are present in the complementary probes.

8, is the 3 x 18 atom spacer region

9, is site for formation of the MTRF amplification complex.

10, is the polypyrimidine region of the MTRF 1 probe

11, is the other polypyrimidine region of the MTRF 1 probe

12, is the reverse polarity of the two polypyrimidine initiator oligonucleotides.

A second embodiment for detection of DNA target sequences is shown in Figures 21A-21B. The numbers refer to the same structures as Fig. 20

Step 1: DNA is isolated and can be subjected to restriction enzymes or physical shearing if necessary.

Step 2: The DNA is denatured using techniques known to those skilled in the art, such as with heat or alkaline conditions.

Step 8: An optional step of UV radiation if photopsoralen is incorporated in the probes, to stabilize the structures formed.

Step 9: Add the biotinylated poly dT signal generation probes. Preferably, these probes are approximately 40 nucleotides. These probes bind to the poly dA regions in the MTRF complex to form the duplex and triplex signal regions.

Step 10: Wash to remove unbound poly dT-biotin probes.

Step 11: Add Streptavidin/Aquorin conjugate in sufficient amount to bind to the biotin molecules.

Step 12: Wash to remove unbound conjugate.

Step 13: Add calcium to activate the aquorin light production and detect signal using a luminometer.

Numbers used in Figure 20 are as follows:

1, is the denatured DNA

2, is the polypurine target region

3, is the duplex forming primary capture probe characterized as having a primary amine or any other biochemical hook attached to the 3' or 5' end, preferably using a spacer molecule.

4, is the spacer molecule.

5, a primary amine is the biochemical hook and binds to N-oxysuccinimide (NOS) coated plates, etc.

6, is the MTRF initiator probe.

7, is the 5' end of the MTRF probe that binds to the 4 base varying sequence of the target sequence. All four bases are present in the complementary probes.

8, is the 3 x 18 atom spacer region

9, is site for formation of the MTRF amplification complex.

10, is the polypyrimidine region of the MTRF 1 probe

11, is the other polypyrimidine region of the MTRF 1 probe

12, is the reverse polarity of the two polypyrimidine initiator oligonucleotides.

A second embodiment for detection of DNA target sequences is shown in Figures 21A-21B. The numbers refer to the same structures as Fig. 20

Step 1: DNA is isolated and can be subjected to restriction enzymes or physical shearing if necessary.

Step 2: The DNA is denatured using techniques known to those skilled in the art, such as with heat or alkaline conditions.

Step 3: Hybridize one strand of the DNA with an aminated (a probe with a primary amine) capture probe that binds antiparallel to the capture region in the target region, forming a duplex structure. The capture probe has a primary amine attached via a spacer region, preferably an 18 atom spacer.

Step 4: Hybridze the single stranded target sequence with another probe that is complementary to a second sequence in the target region to form a second duplex region. Again, this probe binds in an antiparallel direction. After hybridization, a single strand specific nuclease can be added to remove single stranded DNA.

Step 5: A four section MTRF initiator probe is added. The four sections of the probe include a 2 base varying region of the probe that is polypyrimidine rich that binds to the target sequence in parallel to the target (W) sequence, a spacer region, and two polypyrimidine regions joined at the 3' or 5' end to each other. The probe hybridizes to the target region via complementary sequence region of the probe, and binds via triplex formation in parallel fashion to the target (W) sequence. The other regions of the MTRF initiator probe are as previously described.

Step 6: Wash to remove unbound probes.

Step 7: Add the MTRF complex of probes, preferably MTRF 2-8, to generate the MTRF signal amplification system.

Step 8: Wash to remove unbound probes.

Step 9: An optional step of UV radiation if photopsoralen is incorporated in the probes, to stabilize the structures formed.

Step 10: Add the biotinylated poly dT signal generation probes. Preferably, these probes are approximately 40 nucleotides. These probes bind to the poly dA regions in the MTRF complex to form the duplex and triplex signal regions.

Step 11: Wash to remove unbound poly dT-biotin probes.

Step 12: Add Streptavidin/Aquorin conjugate in sufficient amount to bind to the biotin molecules.

Step 13: Wash to remove unbound conjugate.

Step 14: Add calcium to activate the aquorin light production and detect signal using a luminometer.

A third embodiment of the present invention using DNA is shown in Fig 22.

Step 1: DNA is isolated and can be subjected to restriction enzymes or physical shearing if necessary.

Step 2: Hybridize the ds DNA target with a Triplex Forming Oligonucleotide (TFO) probe that functions as the capture probe. The TFO capture probe contains a primary amine as a biochemical hook to allow capture to occur, preferably at the 5' end a spacer region is followed by a primary amine. Preferably, the TFO has a polypyrimidine region that hybridizes to a polypyrimidine/polypurine rich section of the target dsDNA.

Step 3: A four section MTRF initiator probe is added. The four sections of the probe include a 2 base varying region of the probe that is polypyrimidine rich that binds to another target sequence in parallel to the target sequence, a spacer region, and two polypyrimidine regions joined at the 3' or 5' end to each other. The probe hybridizes to the target duplex region via complementary hybridization, and binds in a parallel fashion to the target (W) strand. The other regions of the MTRF initiator probe are as previously described.

Step 4: Wash to remove unbound probes.

Step 5: Add the MTRF complex of probes, preferably MTRF 2-8, to generate the MTRF signal amplification system.

Step 6: Wash to remove unbound probes.

Step 7: An optional step of UV radiation if photopsoralen is incorporated in the probes, to stabilize the structures formed.

Step 8: Add the biotinylated poly dT signal generation probes. Preferably, these probes are approximately 40 nucleotides. These probes bind to the poly dA regions in the MTRF complex to form the duplex and triplex signal regions.

Step 9: Wash to remove unbound poly dT-biotin probes.

Step 10: Add Streptavidin/Aquorin conjugate in sufficient amount to bind to the biotin molecules.

Step 11: Wash to remove unbound conjugate.

Step 12: Add calcium to activate the aquorin light production and detect signal using a luminometer.

In Figure 22 the numbers refer to the following structures:

1, is the DNA

2, is the capture probe. Preferably, the Triplex forming oligonucleotide (TFO) has a polypyrimidine region and binds parallel to a polypurine W, strand of the target. The probe preferably contain a biochemical hook (primary amine) for capture, in this illustration, a spacer region followed by a primary amine.

3, is the spacer region, possibly an 18 atom spacer.

4, is the primary amine group binding to N-oxysuccinimide coating on a solid support.

5, is the MTRF 1 initiator probe

6, is the polypyrimidine region binding parallel to (W) strand of the DNA duplex target.

7, the spacer region, 3x18 atom spacer molecules.

8, is the initiator probe site for formation of the MTRF complex

9, the first polypyrimidine region of the initiator probe.

10, is the second polypyrimidine region of the initiator probe.

11, the reversal of polarity is shown.

Detection of RNA

The MTRF complexes can be used to provide signal amplification for the detection of RNA. All types of RNA are contemplated by the present invention and mRNA will be used for illustrative purposes only. The present invention is used for analysis of a nucleic acid sample for the presence of target nucleic acid sequences. Preferably, the methods of amplification of signal are not limited by the size of the sample, nor by the use of restriction processes, either chemical or enzymatic.

One embodiment of the present invention comprises the following steps.

Step 1: the RNA is isolated. Milligram amounts of nucleic acid can be tested.

Step 2: Restriction of the target sequence can occur. If mRNA is the nucleic acid form of the target sequence, no restriction is required. The capture probe is added. The capture probe can be any probe having a sequence that is complementary to and hybridizes to a portion of the RNA molecule. Most preferably, the capture probe has a primary amine which is connected to the probe via a spacer region, preferably an 18 atom spacer.

Step 3: The MTRF 1 initiator probe is added to the RNA/probe complex of Step 2. One or more MTRF 1 probes may be added at this step. The MTRF 1 probe has a sequence that is complementary to and hybridizes to the RNA target in a different region than the capture probe, although both, in a preferred embodiment should be present the specific target region sought.

Step 4: Wash to remove unbound probes.

Step 5: Add the MTRF complex of probes, preferably MTRF 2-8, to generate the MTRF signal amplification system.

Step 6: Wash to remove unbound probes.

Step 7: An optional step of UV radiation if photopsoralen is incorporated in the probes, to stabilize the structures formed.

Step 8: Add the biotinylated poly dT signal generation probes. Preferably, these probes are approximately 40 nucleotides. These probes bind to the poly dA regions in the MTRF complex to form the duplex and triplex signal reporters.

Step 9: Wash to remove unbound poly dT-biotin probes.

Step 10: Add Streptavidin/Aquorin conjugate in sufficient amount to bind to the biotin molecules.

Step 11: Wash to remove unbound conjugate.

Step 12: Add calcium to activate the aquorin light production and detect signal using a luminometer.

Preferably, this amplification system uses polythymidine oligonucleotides that have biotin molecules attached at approximately every 8 nucleotides. These biotin molecules then react with streptavidin/Aquorin conjugates. Then addition of calcium causes the aquorin molecule to give off light which is detected in a luminometer. Preferably, a duplex reporter complex provides 24 biotin molecules to a 240 nucleotide poly A region. If the duplex reporter complex does not provide sufficient signal for detection, a triplex reporter complex can be formed in which 48 biotin molecules are available for reaction with the streptavidin/aquorin. Table 4 illustrates such numbers.

Table 4

Number of MTRF Complexes	Number of Biotin molecules available	Minimal Target Detection
1	1,000	300
2	5,000	60
3	13,000	23
4	25,000	15
5	41,000	7
6	61,000	5

An embodiment of the MTRF signal amplification process with RNA is illustrated in Figure 23. The numbering in Figure 23 is as follows. Figure 23 shows mRNA which is shown for illustrative purposes only, as other RNA species are contemplated by the present invention.

1, is the 3' poly A mRNA end

2, is the 3' non-coding region

3, is the 5' coding region

4, is the capture probe complementary to any subspecies specific region of the mRNA

5, is a spacer region, preferably three 18 atom spacer that is equivalent in length to a 12 cytosine oligonucleotide.

6, is a primary amino group conjugated to the capture probe by a spacer region.

7, is the 5' end cap of mRNA.

8, is the spacer region that jins the reporter and initiator segments of the secondary initiation probe.

9, is the initiation site for MTRF formation that forms the primary MTRF nucleus that is capable of further reaction with the other MTRF probes.

10, is the first polypyrimidine region of the initiator probe with a polarity that reacts with probes MTRF 2, 3, 4 and 5 to form the primary nucleus.

11, is the reverse polarity of the reporter/initiator probe.

12, is the second polypyrimidine region at the intiator probe with reversed polarity that interacts with probes MTRF 5 and MTRF 2

A second embodiment of the present invention used with RNA is illustrated in Figures 24. The numbers refer to the same structures as Figure 23.

Step 1: the RNA is isolated. Milligram amounts of nucleic acid can be tested.

Step 2: Restriction of the target sequence can occur. If mRNA is the nucleic acid form of the target sequence, no restriction is required. The capture probe is added. The capture probe can be any probe having a sequence that is complementary to and hybridizes to a subspecies specific portion of the RNA molecule. More preferably, the capture probe has a primary amine which is connected to the probe via an 18 atom spacer molecule.

Step 3: For mRNA having a sequence of polypurines, hybridize a polypyrimidine probe, the mRNA having a polypurine sequence in the target region, hybridize a duplex forming polypyrmidine probe. This is achieved to facilitate binding of the TFO reporter/initiator probe.

Step 4: The MTRF 1 initiator probe is added to the RNA/probe complex of Step 2. One or more MTRF 1 probes may be added at this step. The MTRF 1 probe has a sequence that is complementary to and hybridizes to the RNA or the capture probe.

Step 5: Wash to remove unbound probes.

Step 6: Add the MTRF complex of probes, preferably MTRF 2-8, to generate the MTRF signal amplification system.

Step 7: Wash to remove unbound probes.

Step 8: An optional step of UV radiation if photopsoralen is incorporated in the probes, to stabilize the structures formed.

Step 9: Add the biotinylated poly dT signal generation probes. Preferably, these probes are approximately 40 nucleotides. These probes bind to the poly dA regions in the MTRF complex to form the duplex and triplex signal regions.

Step 10: Wash to remove unbound poly dT-biotin probes.

Step 11: Add Streptavidin/Aquorin conjugate in sufficient amount to bind to the biotin molecules.

Step 12: Wash to remove unbound conjugate.

Step 13: Add calcium to activate the aquorin light production and detect signal using a luminometer.

The following is a further embodiment of an MTRF self-complexing probe complex. The probes. described herein, can be readily synthesized in accord with current oligonucleotide synthesis techniques. The probes will be represented by the following sequences but this is not to preclude the use of any other sequences that function similarly. The MTRF 2500 embodiment probes are presented as follows and can be seen in Figure 25, and the sequences used are in Table 5.

Table 5: MTRF / 2500 EMBODIMENT / PROBES

MTRF 1:	(3'...X...3')
5'- Complementary to unique sequence on PDTP or PNAS-X-TTC.TTC.TCT.CTT.CTC.CTC-5'	

MTRF 2:	(3'...X...3')
5'-TTC.TTC.TCT.CTT.CTC.CTC- Poly dA (500mer)-X-Poly dA (500mer)-CTT.TCT.CTC.TTC.CTC.CCT-5'	

MTRF 3:	(5'...X...5')
3'-AGG.GAG.GAA.GAG.AGA.AAG-Poly dA (125mer)-X-Poly dA (125mer)-GAA.GAA.GGA.AAG.AAG.AGG.-3'	

MTRF 4:	(3'...X...3')
5'-CCT.CTT.CTT.TCC.TTC.TTC- Poly dA (500 mer)-X- Poly dA (500 mer)-TCT.TCC.TTC.TTC.CTC.TTT-5'	

MTRF 5:	(5'...X...5')
3'-AAG.AAG.AGA.GAA.GAG.GAG- Poly dA (125 mer)-X-Poly dA (125 mer)-AGA.AGG.AAG.AAG.GAG.AAA-3'	

MTRF 6:	(3'...X...3')
5'-CTT.CTT.CCT.TTC.TTC.TCC-X-TTC.TTC.TCT.CTT.CTC.CTC-5'	

MTRF 7:	(3'...X...3')
5'-TCT.TCC.TTC.TTC.CTC.TTT-X-TCC.CTC.CTT.CTC.TCT.TTC-5'	

MTRF 8:	(3'...X...3')
5'-CTT.CTT.CCT.TTC.TTC.TCC-X-TTC.TTC.TCT.CTT.CTC.CTC-5'	

MTRF 9:	(3'...X...3')
5'-TCT.TCC.TTC.TTC.CTC.TTT-X-TCC.CTC.CTT.CTC.TCT.TTC-5'	

MTRF 1: A probe comprised of two oligonucleotides joined at the 3' end usually with an accompanying reverse of polarity. This probe is referred to as the initiator probe, having one 5' end complementary to a unique sequence on the PNAS or PDTP complexes, a 3x18 atom spacer, and two polypyrimidine sequences (~18 mer each) with a polarity reversal between them. The oligos are joined by any method known to those skilled in the art.

MTRF 2: A probe comprised of two oligonucleotids joined at the 3' end usually with an accompanying reverse of polarity. This probe is a combination of two probes posessing a 5' polypyrimidine sequence and a 3' poly dA sequence which will provide the core structure for signal amplification. The two probes are joined at the 3' end by any method known to those skilled in the art.

MTRF 3: A probe comprised of two oligonucleotides joined at the 5' ends usually with an accompanying reverse of polarity. This probe is a combination of two probes possessing a 3' polypurine sequence and a 5' poly dA sequence which will provide the core structure for signal amplification. The two probes are joined at the 5' end by any method known to those skilled in the art.

MTRF 4: A probe comprised of two oligonucleotides joined at the 3' ends usually with an accompanying reverse of polarity. This probe is a combination of two probes posessing a 5' polypyrimidine sequence and a 3' poly dA sequence which will provide the core structure for signal amplification. The two probes are joined at the 3' end by any method known to those skilled in the art.

MTRF 5: A probe comprised of two oligonucleotides joined at the 5' ends usually with an accompanying reverse of polarity. This probe is a combination of two probes possessing a 3' polypurine sequence and a 5' poly dA sequence which will provide the core structure for signal amplification. The two probes are joined at the 5' end by any method known to those skilled in the art.

MTRF 6: A probe comprised of two oligonucleotides joined at the 3' ends usually with an accompanying reverse of polarity. This probe is a combination of two probes possessing a polypyrimidine sequence. The two probes are joined at the 3' ends by any method known to those skilled in the art.

MTRF 7: A probe comprised of two oligonucleotides joined at the 3' ends usually with an accompanying reverse of polarity. This probe is a combination of two probes possessing a polypyrimidine sequence. The two probes are joined at the 3' end by any method known to those skilled in the art.

MTRF 8: A probe comprised of two oligonucleotides joined at the 3' ends usually with an accompanying reverse of polarity. This probe is a combination of two probes possessing a polypyrimidine sequence. The two probes are joined at the 3' end by any method known to those skilled in the art.

MTRF 9: A probe comprised of two oligonucleotides joined at the 3' ends usually with an accompanying reverse of polarity. This probe is a combination of two probes possessing a polypyrimidine sequence. The two probes are joined at the 3' end by any method known to those skilled in the art.

Another embodiment of the MTRF nucleus structure is presented in Figure 26, wherein the triplex regions of the MTRF nucleus structure is stabilized by psoralen cross-links. The numerical designations for the MTRF nucleus shown in Figure 26 are as follows:

1, the MTRF 1 initiator probe consisting of a probe with two 3` ends (reverse polarity) separated by a linker or spacer molecule that has one 3' end complementary to sites on the MTRF co-initiator probe and another 3` end complementary to one arm of the MTRF "nuleus".

2, the MTRF 2 probe is composed of two 5' polypyrimidine ends each of unique sequence joined by a poly dA region with a reversal of polarity in this region.

3, the MTRF 3 probe is composed of two 3' polypurine ends each of unique sequence joined by a poly dA region with a reversal of polarity in this region.

4, the MTRF 4 probe is composed of two 5' polypyrimidine ends each of unique sequence joined by a poly dA region with a reversal of polarity in this region.

5, the MTRF 5 probe is composed of two 3' polypurine ends each of unique sequence joined by a poly dA region with a reversal of polarity in this region.

6, the MTRF 6 probe with 3 sections, one, a unique GC rich region on the 3' end, two, a short spacer, and three, a unique polypyrimidine sequence on the 5' end forming a triplex with MTRF 2 and MTRF 5.

7, the MTRF 7 probe with 3 sections, one, a unique GC rich region on the 3' end, two, followed by a short spacer, and three, a unique polypyrimidine sequence on the 5' end forming a triplex with MTRF 2 and MTRF 3.

8, the MTRF 8 probe with 3 sections, one. a unique GC rich region on the 3' end, two, followed by a short spacer, and three, a unique polypyrimidine sequence on the 5' end forming a triplex with MTRF 3 and MTRF 4.

9, the MTRF 9 probe with 3 sections, one. a unique GC rich region on the 3' end, two, followed by a short spacer, and three, a unique polypyrimidine sequence on the 5' end forming a triplex with MTRF 4 and MTRF 5.

10, are 3' polypurine end regions of the MTRF-3 and -5 probes, each possess different sequence specificities. Note that 8-aminoadenosine and 8-aminoguanosine are substituted for adenosine and guanosine in these regions.

11, are two antiparallel polypyrimidine strands, of identical sequence, form a triplex region with the four unique polypurine sequences of MTRF-3 and -5. Again, 5-methylcytosine can be substituted in the polypyrimidine TFO to stabilize the nucleus at pH values approaching 7.0 (optimum triplex stability is at pH 5.5).

12, is the reversal of polarity in probes MTRF-2, -3, -4, and -5.

13, are polydA regions that form a core for label introduction, via the duplex (polydA - polydT) reporter and triplex reporter (polydT – polydA - polydT).

14, is the biotinylated polydT - polydA duplex reporter present on all four probes MTRF-2, -3, -4, and -5.

15, is the biotinylated polydT – polydA - polydT triplex reporter present on all four probes MTRF-2, -3, -4 and -5.

16, are short spacers within probe MTRF-6, -7,-8 and -9.

17, are GC-rich 3' ends of probes MTRF-6, -7, -8 and –9, that bind the complementary regions on the MTRF-1 initiator, and the other MTRF nuclei, thereby initiating formation of a lattice structure attached to the MTRF co-initiator probe.

18, are psoralen crosslinks, mediated by the introduction of dC-photopsoralen, found in the four polypyrimidine triplex formation regions that stabilize the MTRF nucleus. This is achieved by locking the triplex regions by covalently crosslinking all 3 strands of the triplex, creating stable MTRF nucleus box structures.

The above embodiment of MTRF signal amplification is easily pre-assembled and thus, reduces the complexity of the assay.

Immuno-MTRF

The present invention comprises methods and compositions comprising use of the MTRF probe complex signal amplification system for the detection of antigens, with high sensitivity,

through the attachment of MTRF probes to a target antigen-specific monoclonal or polyclonal antibody. The MTRF signal amplification system can be used in any antibody assay that is known to those skilled in the art, preferably formats for assays are similar to ELISA formats, hereinafter, immunoassays. These methods and compositions, herein referred to as Immuno-MTRF, allow the detection of exceedingly low numbers of antigen molecules, that might otherwise be undetectable by established assay procedures. Though the MTRF can be bound to the antibody or other binding partner through any method, it is preferable to use the co-initiator probe. The co-initiator probe for the MTRF complex is directly linked to any region of an antibody molecule other than the antigen-binding site. Preferably, linking will be to the Fc region of the antibody, and will initiate formation of the MTRF signal amplification cascade. The present invention contemplates the use of Immuno-MTRF compositions and methods in any ligand/co-ligand assay where there are specific binding partners and detection of the binding can be accomplished through a label or amplification system of labeling. Antibody-antigen interactions are described herein, but other specific binding partners are contemplated in such examples.

The antibody-DNA conjugates of the Immuno-MTRF embodiments allow multiple labels of any type to be bound to a target antigen by repeated rounds of MTRF formation. Signal amplification by such means increases the sensitivity of detection of the targeted antigen, down to very low levels. Additionally, since the expression of most proteins in a single cell results in production of many thousands of identical protein molecules per expressed gene, attaching thousands of labeled entities to each antigen/antibody complex will enhance the signal amplification, giving a highly sensitive assay that will permit the detection of single target cell.

This hybrid technology combines the molecular recognition and specificity inherent in antibodies or other specific binding partners, with the high signal amplification potential of the MTRF system. The sensitivity of the Immuno-MTRF assay system allows for the detection of a single cancer or pathogen cell, and can be used in assays to determine residual disease, viral load, and early stage cancer metastasis.

The current ELISA assays can suffer from limitations in their usefulness because of antigen cross-reactivity, and the inability to detect low numbers of antigen/antibody complexes. While the advent of monoclonal antibodies has reduced problems arising from cross-reactivity, however, the low sensitivity inherent to these assays is still a practical impediment to their use under certain circumstances.

The Polymerase Chain Reaction, and other DNA amplification-based technologies, have been used to improve the sensitivity of the immunoassay. This immuno-PCR hybrid of protein

and DNA primers did offer an improvement in the area of sensitivity. It suffered from other disadvantages concerned with the sensitivity and specificity of the PCR reaction, and raised additional issues of the possibility of contamination, and the overall complexity of the assay system.

In general, the methods and compositions of the Immuno-MTRF embodiments comprise combining monoclonal antibodies with a self-complexing probe signal amplification system. This is accomplished by directly or indirectly attaching MTRF signal amplification probes to the antigen-specific antibody molecule or other specific binding partner. In one embodiment, the MTRF initiator probe is directly bound to an antibody molecule and functions like the co-initiator probe to initiate the formation of the MTRF Complex. The MTRF probes are added to form the nuclei as shown in previous Figures. The MTRF initiator probe can be attached to a primary (directly bound to the antigen) or secondary antibody (bound to an antibody that binds another antibody) and functions as described below.

In another embodiment of the Immuno-MTRF assay system, a MTRF co-initiator probe is directly attached to the specific monoclonal antibody directed to the antigenic target. This is exemplified in Figure 27. In another embodiment, the MTRF co-initiator is attached to an antibody that binds to an antibody bound to the antigen such as an anti-mouse IgG, anti-sheep IgG, or anti-goat IgG, etc. This embodiment allows flexibility in the choice of the source of the primary antibody directed against a particular target antigen. The antibody linked to the MTRF will be selected to recognize antibodies from the specific species that was the source of the target-specific primary antibody. See Figure 29. In other words, in a sandwich immunoassay, the monoclonal antibody specific for the target antigen can be directly linked to an MTRF co-initiator probe. Alternatively, if the monoclonal IgG is of mouse origin, then an anti-mouse IgG raised in a different species can be similarly modified by linking to an MTRF co-initiator, and will then bind to the antigen/mouse antibody complex. The target antigen can be detected using the highly sensitive MTRF signal amplification system. Additionally, any reacting antibody or specific binding partner can be labeled using such methods and compositions.

In a preferred embodiment, the MTRF complex co-initiator probe may be bound to an antibody by means of any sulfide or other reactive moiety on the antibody molecule via a homobifunctional alkylating agent. This would result in a covalent crosslink between an amino group at one end of the MTRF co-initiator, and a sulfhydryl group on a cysteine residue of the antibody molecule at a site other than in the antigen-binding region.

In general, the Immuno-MTRF embodiments comprise modifying the reporter antibody with an initiator MTRF probe and formation of the MTRF complexes or modifying the antibody

with a co-initiator probe and the subsequent addition of an initiator probe, and MTRF nucleus complexes. This results in the ultimate attachment of multiple detectable labels to the antigen/antibody-DNA complex. The procedural complexity of the MTRF "nuclei" addition is significantly reduced compared to alternative signal and DNA amplification diagnostic schemes. This present invention can be used for the simultaneous detection of multiple antigen targets in the same sample.

The label that is detected to signal the target antigen/antibody complex-MTRF complex can be attached directly to the MTRF 'nucleus', or indirectly. In indirect methods, the signaling means is achieved by attaching a suitable ligand to the MTRF structure, followed by the addition of a ligand-specific molecule, most probably but not limited to, an antibody bearing a labeling means. Such a label could be a suitable enzyme, or any other labels known to those skilled in the art.

An embodiment of the Multiple Triplex Reporter Formation (MTRF) signal amplification system, comprising the following steps, leads to generation of signal in immunoassays. The following example illustrates use of a co-initiator probe. It is to be understood that the MTRF initiator probe can be bound covalently or through other means to the antibody.

Step I: Covalently attach the co-initiator probe to the antibody molecule at a site other than the antigen-binding site. This is mediated by interaction of a sulfhydryl group on the antibody, with a primary amine moiety at the 3' end of the MTRF co-initiator probe. The co-initiator probe can be of any length.

Step II: Isolate a sample containing the target antigen by any method known to one skilled in the art. The sample can be any source that contains the target antigen, including but not limited to bodily samples from humans or animals, plant-derived antigens, environmental sources or undetermined sources.

Step III: Add the antibody/co-initiator complex to the sample to be screened for the presence of the target antigen.

Step IV: Capture the analyte/antibody/MTRF co-initiator complex by attaching it to a solid support. In one embodiment, this may be achieved by binding a second, different, antibody to a solid substrate such as a microtiter plate wall. This second antibody has specificity to a second epitope on the target antigen, and is a means to attach the target antigen/initial antibody/MTRF co-initiator complex to the solid support, similar to the familiar ELISA sandwich immunoassay.

Step V: Wash to remove unbound antibody/co-initiator complexes

Step VI: The 3' region of the MTRF-1 initiator probe includes a GC-rich region that complements the MTRF co-initiator GC rich sequence, a linker between the oligonucleotides, and a GC-rich region complementary to one GC-rich arm of the MTRF nucleus structure. Hybridization of the initiator probe and co-initiator is optimal at room temperature, due of the high GC content, and spontaneously under conditions where an excess of initiator probes to co-initiator probe sites is used.

Step VII: Wash to remove excess MTRF initiator probe.

Step VIII: Initiate MTRF signal cascade formation by hybridizing with at least an equal number of MTRF nuclei as there are multiple possible binding sites of MTRF initiator probes. This will provide all of the potential MTRF complex initiator sites with at least one MTRF nucleus.

Step IX: Hybridize with an excess of MTRF nuclei over the expected number of MTRF structure starts. This secondary MTRF nucleus hybridization will further insure that the maximum number of possible MTRF starts occurs, and that each is continuously built upon until the point is reached where steric hindrance prevents the further addition of MTRF nuclcii.

Step X: Wash to remove unbound probes.

Step XI: Hybridize the complexes that have been formed and attached to the solid support, with a polydT probe containing a multiplicity of label molecules or binding partners that function as labels, preferably biotin. The combination of duplex and triplex reporter functions of the MTRF complex insures that multiple label moieties are attached to the complex. The present embodiment contemplates biotin-streptavidin labeling binding partners, but any label known to those skilled in the art can be used with the present invention.

Step XII: Wash to remove unbound poly dT-biotin signal amplification probes.

Step XIII: Add a streptavidin-enzyme conjugate. The streptavidin has high affinity for the biotin molecules associated with the MTRF complexes, thereby attaching multiple streptavidin-enzyme molecules to the MTRF complexes. Other embodiments will vary as to the labeling system used, and may include direct dye labeling of the MTRF, labeled oligonucleotides, or attachment of a dye by means of a streptavidin-dye conjugate.

Step XIV: Wash to remove unbound conjugate

Step XV: In the event that streptavidin-enzyme is the label of choice, add an enzyme substrate, and spectrophotometrically determine the level of the enzyme attached to the MTRF-based complex. With a dye, or other non-enzyme label, determine the extent of labeling by any method that is appropriate and known to one skilled in the art.

An embodiment of MTRF introduction onto an antigen-specific antibody to improve the sensitivity of ELISA Assays is presented in Figure 28. The numerical designations in Figure 28 are represented by:

1, a GC-rich MTRF co-initiator probe sequence comprising an amine or similar reactive moiety on the 3' end, and a consecutive series of GC-rich regions (A') 2, of any desired length, each region separated by a spacer 7 with as many repeats of the sequence as necessary for the assay system.

2, a GC-rich MTRF co-initiator probe sequence (A') complementary to one of the initiator probe GC-rich regions (A).

3, a MTRF initiator probe composed of three sections, a GC-rich sequence (A) at one 3' terminus and complementary to that (A') found on the co-initiator probe, 5, another GC-rich region complementary to a region of the MTRF nucleus 9 and at the opposite 3' end and a spacer or linker 6, such as is known to those skilled in the art. In other embodiments, 3 may attached directly to the antibody through 8.

4, GC-rich sequence A, complementary to certain regions of the MTRF co-initiator probe.

5, GC-rich region B of the MTRF initiator probe that is complementary to a region on the MTRF nucleus structure 9.

6, spacer or linker, of any desired length, of the MTRF initiator probe.

7, spacer or linker, of any desired length, of the MTRF co-initiator probe.

8, covalent crosslink between the primary target antigen-specific antibody, and the MTRF co-initiator probe, comprising a '-NH2-linker-S-' bridge.

9, MTRF nucleus structure consisting of a number of self-hybridizing oligonucleotide probes that together form a lattice structure to permit the introduction of a multiplicity of labels to the target antigen/probe complex.

10, region of an MTRF probe that complements a region on the initiator probe, and other MTRF nuclei probes, for the initiation and continuation of the nucleus binding.

An embodiment of a MTRF label amplification system wherein an antibody-based assay format is used to detect an antigen is presented in Figure 27. Numerical designations in Figure 27 are:

1, capture antibody bound to a solid support.

2, protein or other immunogenic target

3, reporter antibody with an MTRF co-initiator probe attached, via a chemical crosslink, to a site not within the antigen-binding region.

4, crosslinking means, e.g., homobifunctional alkylating agent chemical crosslink.

5, spacer

6, repetitive GC-rich region on the MTRF co-initiator that is complementary to a specific GC-rich region on the MTRF initiator probe.

7, primary MTRF nucleus

8, MTRF initiator probe with 3 sections, a GC-rich region at one 3' end, 10, and complementary to the GC-rich regions on the MTRF co-initiator probe, a spacer, 11, and a second GC-rich region at the opposite 3' end, 9 that complements to a unique region, 9, on the MTRF nucleus structure, B'.

9, GC-rich region with a unique sequence that complements a site on the MTRF nucleus.

10, GC-rich region with a unique sequence complementary to a site on the MTRF co-initiator probe.

11, spacer (in this particular embodiment a short spacer) that provides matrix flexibility.

12, MTRF nucleus structure.

13, solid support

Yet another embodiment of the MTRF-enhanced antibody assay, wherein the antibody linked to the MTRF is a species-specific anti-IgG antibody, and the target antigen-specific primary antibody is linked to a solid support. The numerical designations in Figure 29 are:

1-13, as in Figure 27

14, species-specific anti-IgG antibody with attached co-initiator probe.

The present invention also contemplates the use of multiple specific antibodies that are detected using MTRF methods and compositions. Such multiplexing assays can be achieved by the use of a combination of monoclonal antibodies with different antigen specificities, each of which is conjugated to an MTRF initiator probe to begin the MTRF cascade formation.

The Immuno-MTRF mediated signal detection of antigenic targets includes advantages such as the use of specificity of antibodies, particularly monoclonal antibodies. The MTRF embodiments offer a large number of labels to be attached to the antigen/antibody-MTRF complex, compared to other signal amplification systems currently available that initiate multiple complex formations at different sites on a target, each with a limited capability to attach few labels to the target. The MTRF probes are self-complexing so label attachment is supported by the integrity (stability) and growth of the MTRF probes complex.

Additionally, the present invention can be used to detect the presence of cancer or abnormal cell growth in the body. Some tumor markers are proteins that express themselves only in the cancer state, and not in normal cells. Increasing detection of such proteins using the methods and compositions of Immuno-MTRF embodiments would provide evidence of the

cancer state. Other uses for Immuno-MTRF embodiments include diagnosis of antigenic targets earlier in the infectious timecourse of a disease. Such early detection provides treatment options to the patient that may not be available at later times. Furthermore, Immuno-MTRF can be used as an adjunct to nucleic acid diagnostics in determining pathogenic states.

BROMOURACIL LINKED BASES – BU CUTTER PROBE TECHNOLOGY

The present invention comprises methods and compositions comprising the use of a 5-bromouracil (5BU)-containing oligonucleotide Cutter Probe (CP) for the restriction cleavage of single or double strand DNA or RNA, at any desired position along a target sequence, and which is independent of enzyme digestion of the target nucleic acid. These methods and compositions avoid the cleavage site limits imposed by the sequence specificity inherent to restriction enzymes. Both the *in vitro* cleavage, and the therapeutic application to *in vivo* cleavage of nucleic acid by the 5BU-modified Cutter Probes of the present invention, are contemplated.

The present invention contemplates a single strand Cutter Probe that includes 5BU-modified bases to increase the double strand break frequency (the target restriction frequency). Most preferably, 5BU molecules are attached, via linker molecules, to certain positions on any of the bases cytosine, thymine, adenine, or guanine, and base analogs, or other DNA binding ligands, including, but not limited to, polyamine hairpins.

One embodiment of the present invention is a 5BU-containing probe contain 5-bromouracil as a substitute base only, replacing nucleotides incorporated in the nucleic acid sequence. With this embodiment, there is a high frequency of single strand breaks within the cutter probe itself, but a lower (<1%) frequency of breaks of the phosphate sugar backbone of the opposite target nucleic acid strand. As contemplated by the present invention, the introduction of 5BU attached by linkers, to any of the bases of a nucleic acid sequence, and their insertion by intercalation in adjacent base stacking planes, allows free radicals to be formed closer to the sugar-phosphate backbone of the opposing target strand, and a much improved frequency of strand scission will result. Preferred 5BU modifications of nucleotide bases incorporated in a nucleic acid sequence are summarized in Table 6:

Table 6

MODIFIED BASE	Position Attached On Nucleotide Base	Position Attached On BU Molecule
CYTOSINE (BU)	C 4	N 1
THYMINE (BU)	C 5	N 1
ADENINE (BU)	C 8	N 1
GUANINE (BU)	C 8	N 1

Any linker molecule known to one skilled in the art may be used providing that it will ultimately juxtapose the intercalating 5BU moiety of the modified base to the phosphate sugar backbone of the opposite, target, strand. The 5BUs that substitute for bases within the cutter probe sequence itself, and the 5BU groups that are linked to other bases of the cutter probe, will, therefore, deliver multiple 5BU molecules to the adjacent base stacking planes. Upon activation by irradiation, the concentration of 5BUs will form free radicals that will cleave a defined and restricted section of the target nucleic acid strand, by disruption of the phosphate-sugar backbone/s. The 5BU-containing region of the probe itself will also be destroyed.

In a preferred embodiment, the number of 5BU-linked bases that may be incorporated, and therefore placed by intercalation in close proximity to the sugar-phosphate backbone of the opposite strand, is limited solely by the size of the Cutting Probe oligonucleotide. Consequently, there is no limit to the number of uracilyl free radicals that could be brought to bear to destabilize the opposite (target) phosphate-sugar backbone, and generate the required double strand breaks therein.

Figure 30 depicts a preferred embodiment of the 5BU Cutter Probe that will result in efficient restriction cleavage of a single strand DNA target.

Step I: Obtain a biological sample and isolate the DNA therein by any method known to one skilled in the art. See Fig. 30 (1).

Step II: Heat denature DNA to generate single strand (ss) DNA. See Fig. 30 (2).

Step III: Hybridize with two BU Cutter Probes, BUCP-1 and BUCP-2, to form duplex regions. BUCP-1 has, at its 3' end, any number of 5BU substitutions or 5BU-linked bases (any of A, T, G, or C). BUCP-2 has at its 5' end any number of 5BU substitutions or 5BU-linked bases (any of A, T, G, or C). Both probes may include regions that complement the target sequence, but do not themselves include 5BU containing groups. See Fig. 30 (3). These regions will not, therefore, be disrupted after irradiation, and will remain hybridized to the target nucleic acid. They will then be available for later isolation of the target sequence. See Fig. 30 (3).

After probe hybridization, the base-linked 5BU moieties will preferably intercalate in the base stacking plane, and form multiple bromide and uracilyl free radicals after irradiation. Upon irradiation, the intercalated 5BU molecules connected to bases by preferred extended linkers, will generate free radicals in adjacent base stacking planes that will be close to the sugar-phosphate backbone of the opposite strand. This will destabilize the backbone connecting the oligonucleotide bases of the nucleic acid strand. This means of cleavage is applicable to both Watson or Crick strands of DNA, and to ssRNA. Another preferred embodiment is contemplated, but not limited to, a Triplex Forming Oligonucleotide (TFO) used to restriction cleave a non-denatured double strand (ds) DNA, or dsRNA.

Step IV: The restriction cleaved target may be isolated from the digestion mix by any method known to those skilled in the art, including affinity molecules. The affinity molecules can be attached to BUCP-1 or BUCP-2 in any region other than the restriction region itself, which will have been disrupted by the formation of the 5BU free radicals. In one preferred embodiment, affinity molecules such as, but not limited to, a primary amine on the probe BUCP-1 or BUCP-2, can bind to a solid support coated with n-oxysuccinimide (NOS). See Fig. 30 (4)

The numerical designations in Figure 30 represent:

1, native genomic DNA, including ds target

2, single stranded (ss) DNA target

3, restriction regions, bordering the target, 2, that may be disrupted by free radical formation, usually by the more powerful uracilyl free radical.

4, 5-Bromouracil Cutter Probe BUCP-1 possesses a region complementary to the target nucleic acid, and a region that, when activated by irradiation, will restrict the single strand target, together with any segment of the cutter probe that contains 5BU and which generates the free radicals.

5, 5-Bromouracil Cutter Probe BUCP-2 possesses a region complementary to the target nucleic acid and a region that, when activated by irradiation, will restrict the single strand target, and destroy any segment of the cutter probe that contains 5BU and which generates the free radicals.

6, region a-b, that contains the target sequence, 2, to be restricted, and limited in length solely by the chosen positions of hybridization of the BUCPs.

7, region a' and b' may be used in capture, detection, sequencing, or other methodologies. These regions include the intact remaining sequences of the BUCP-1 and BUCP-2 probes.

8, restricted target, 2, or Protected Nucleic Acid Sequence (PNAS).

9, 5-Bromouracil integrated into the BUCP oligonucleotide as either as the nucleotide 5BU, and/or as natural or modified bases linked to additional 5BU molecules, and which together will subject the adjacent base stacking planes to multiple free radicals, after irradiation.

10, capture mechanism, such as a primary amine that may bind to the n-oxysuccinimide (NOS) coated plate (microtiter) or other solid support

Figure 31 depicts yet another preferred embodiment of the BU-containing Cutter Probes that will result in high frequency restriction cleavage of single strand RNA targets or ds RNA targets.

Step Ia: Obtain a biological sample and isolate single strand mRNA by any method known to those skilled in the art (See Fig. 31 (1a); or

Step Ib: Obtain a biological sample and isolate RNA, and heat denature the double strand (ds) ribosomal RNA (rRNA), thereby generating single strand RNA for restriction cleavage. See Fig. 31 (1b).

Step II: Hybridize with two BU Cutter Probes to form duplex regions. BUCP-1 has at its 3' end any number of 5BUs or 5BU-linked bases (any of A, T, G, or C). BUCP-2 has at its 5' end any number of 5BUs or 5BU-linked bases (any of A, T, G, or C). In one preferred embodiment, one or both probes may include regions that complement the target sequence, but do not themselves include 5BU groups. See Fig. 31 (2). These regions will preferably not be disrupted after irradiation, and will remain hybridized to the target nucleic acid. They will then be available for isolation of the target sequence and identification of the target sequence.

Step III: After probe hybridization, the base-linked 5BU moieties will intercalate in the base stacking plane, and form multiple bromide and uracilyl free radicals after irradiation. Upon irradiation, the intercalated 5BU molecules connected to bases by extended linkers, will generate free radicals, in adjacent base stacking planes, that will be close to the sugar-phosphate backbone of the opposite strand. This will be sufficient to destabilize the backbone connecting the oligonucleotide bases of the nucleic acid strand. In other embodiments contemplated, a Triplex Forming Oligonucleotide incorporating 5BU reactive groups (BUTFO) is used to restriction cleave a non-denatured ds rRNA.

Step IV: The restricted target may be isolated from the solution by any method known to those skilled in the art including, but not limited to, affinity molecules. The affinity molecules can be attached to BUCP-1 or BUCP-2 in any region other than the restriction region itself, which will be disrupted by the 5BU free radicals. See Fig. 31 (3). Alternatively, the affinity molecules can be attached to a BUTFO that forms a triplex region in the region between the

restriction cleavage sites. In one embodiment, affinity molecules such as a primary amine on probe BUCP-1 or BUCP-2, can bind to a solid support coated with n-oxysuccinimide (NOS).

The numerical designations in Figure 31 represent:

1, ss nucleic acid containing the target to be restricted.

2, the target sequence

3, restriction regions, bordering the target, 2, that may be disrupted by free radical formation, usually by the more powerful uracilyl free radical.

4, 5-Bromouracil Cutter Probe BUCP-1 possesses a region complementary to the target nucleic acid, and a region that, when activated by irradiation, will restrict the single strand target, together with any segment of the cutter probe that contains 5BU and which generates the free radicals.

5, 5-Bromouracil Cutter Probe BUCP-2 possesses a region complementary to the target nucleic acid and a region that, when activated by irradiation, will restrict the single strand target, and destroy any segment of the cutter probe that contains 5BU and which generates the free radicals.

6, region a-b, that contains the target sequence, 2, to be restricted, and limited in length solely by the chosen positions of hybridization of the BUCPs.

7, region a' and b' may be used in capture, detection, sequencing, or other methodologies. These regions include the intact remaining sequences of the BUCP-1 and BUCP-2 probes.

8, restricted target, 2, or Protected Nucleic Acid Sequence (PNAS).

9, 5-Bromouracil integrated into the BUCP oligonucleotide as either as the nucleotide 5BU, and/or as natural or modified bases linked to additional 5BU molecules, and which together will subject the adjacent base stacking planes to multiple free radicals, after irradiation.

10, capture mechanism, such as a primary amine that may bind to the n-oxysuccinimide (NOS) coated plate (microtiter) or other solid support

In yet another embodiment of the present invention, shown in Figure 32, the BU Cutter Probes will result in efficient restriction cleavage of a double strand (ds) nucleic acid target (DNA or RNA). See Fig. 32 (1).

Step I: Obtain a biological sample and isolate DNA containing the target nucleic acid sequence to be restriction cleaved by any method known by those skilled in the art.

Step II: Hybridize with one or more Triplex Forming Oligonucleotides (BUTFOs) each of which is capable of restriction cleaving a specific polypyrimidine-rich site (use one probe for two cuts-excision, or one probe with two opposite ends harboring restriction groups for total target excision). Multiple BUTFOs will aid the isolation of specific kilobase segments of the

genomic DNA. They will also provide a means to selectively destroy those genes such as, but not limited to, those associated with targeted cell death, and gene destruction in vitro. The BU Cutter Probes each have two regions, on is a cleavage specific restriction site. The other region can bind to sequences upstream or downstream from the target, or alternatively, can bind to the target nucleic acid sequence. The binding sites can be located anywhere in the genome. The triplex forming element of this embodiment demands that the Cutter Probe sequence be pyrimidine-rich (C or T). The additional 5BUs attached to the C or T bases will intercalate within the base stacking planes, and form bromide and uracilyl free radicals upon irradiation. The length of the linker between a base and the attached 5BU group will dictate where the uracilyl and bromine radicals will be formed within the base stacking plane. See Fig. 32 (2).

Step III: Expose the dsDNA and Cutter Probe complex, (a triplex) to, UV light, x-rays, or gamma-radiation to generate free radicals in the base stacking plane. The free radicals are ideally positioned to intercalate in adjacent base stacking planes and will be sufficient to simultaneously destabilize the phosphate-sugar backbones of the Watson and Crick strands within the duplex DNA target. See Fig. 32 (3).

Step IV: The restricted target may be isolated from the solution by any method known to those skilled in the art including, but not limited to, affinity molecules. The affinity molecules can be attached to BUCP-1 or BUCP-2 in any region other than the restriction region itself, which will be disrupted by the 5BU free radicals. See Fig. 32 (3). Alternatively, the affinity molecules can be attached to a BUTFO that forms a triplex region in the region between the restriction cleavage sites. In one embodiment, affinity molecules such as a primary amine on probe BUCP-1 or BUCP-2, can bind to a solid support coated with n-oxysuccinimide (NOS).

The numerical designations in Figure 32 are represented by:

1, genomic double stranded DNA.

2, the target region.

3, restriction regions composed of multiple free radical-forming bases.

4, 5BU free radical-forming molecules, located near adjacent base stacking planes by integrating 5BU directly into the BUTFO, or by the addition of 5BU to any other base by means of variable length linkers. Both modes of incorporating 5BU will intercalate free radicals into the base stacking planes.

5, variable length linkers.

6, BUTFO-1, with two sections; first, a region linked to 5BU molecules, that is responsible for the free radical destruction of the sugar-phosphate backbones of the target Watson and Crick strands, thereby generating a double strand break; second, a region not subject to destruction and

which is complementary to some region of the target, such as the entire target or just to the ends of the target.

7, BUTFO-2, with two sections, a polypyrimidine-rich (C or T) region linked to 5BU molecules responsible for the free radical destruction of the sugar-phosphate backbones of the target Watson (polypurine), and Crick (polypyrimidine) strands, thereby generating a double strand break, and a second region not subject to destruction and which is complementary to the entire target, or just to the ends of the target.

8, BUTFO probes, multiple BUTFO complexes may be formed, and can be contiguous or separated by multiple bases.

9, desired restriction cleaved target must be in close proximity to, but not integral with, the restriction cleaved region, and each restriction region must form a stable triplex with the BUTFO cutter.

10, hydrogen bonding between the duplex DNA Watson and Crick strands.

11, Hoogsteen's bonding between the BUTFO and the purine-rich strand of the target nucleic acid sequence.

12, target segment restriction cleaved from the genomic DNA, and which comprises the nucleic acid sequence between one BUTFO end, and the end of the opposite BUTFO, minus the lengths of the two restriction regions that were subject to free radical destruction. An affinity molecule may also be attached to the target region, or anywhere within either of the two TFOs other than the restriction regions destroyed by the free radicals.

13, BUTFO-1 with a polypyrimidine-rich region, and a region not subject to destruction and which is complementary to the entire target, or just to the ends of the target.

14, BUTFO-2 with a polypyrimidine-rich region, and a region not subject to destruction and which is complementary to the entire target, or just to the ends of the target.

15, restriction cleaved target dsDNA.

16, capture mechanism, e.g., an amine group that will react with a NOS-coated solid support

Triplex Stability and 8 Aminopurine Substitution

The present invention contemplates methods and uses wherein modified triplex nucleic acid structures can be stabilized at pH 7.0 by the introduction of 8-aminoadenosine (8aA) and 8-aminoguanosine (8aG) to the polypurine strand of the triplex.

Triplex nucleic acid structures occur at pH 5.5, at which pH the cytosine is protonated. Stability of the pyrimidine-purine-pyrimidine triplex at pH 7.0 can be enhanced with the addition of 5-methylcytosine to the TFO to aid in PNAS stability in TPA. This has prompted reduction of

the pH of the assay in order to assist 5mC substituted TFOs to generate enhanced triplex stability. Studies have indicated that the Watson strand of the triplex complex, containing the substituted purines 8aA or 8aG, compares favorably to when 5-methlcytosine (5mC) is introduced into the TFO strand. The use of 8aA or 8aG in the present invention offers the advantage of stabilizing the triplex to a greater extent than does 5mC, at pH 7.0.

Figures 33A–33C show a preferred embodiment of the TPA assay of single strand nucleic acid, either DNA or RNA, wherein the triplex forming oligonucleotide (TFO) includes the modified purine bases 8aA and/or 8aG, and is combined with the MTRF signal amplification system. The method of the preferred embodiment is given by the following steps:

Step I: Obtain a biological sample and isolate the nucleic acid (DNA or RNA) therein by any method known to those skilled in the art.

Step II: Denature the nucleic acid to single strand form by use of heat, mild alkali, or any other method known to those skilled in the art and which does not degrade the nucleic acid.

Step III: (First Level of Specificity) Hybridize the single strand polypyrimidine target nucleic acid sequence within the isolated nucleic acid, with a reverse polarity triplex forming oligonucleotide (RP-TFO) having a polypurine region complementary, and therefore antiparallel, to the polypyrimidine target. The RP-TFO will include the modified purine bases 8-aminadenosine (8aA) and 8-aminoguanosine (8aG). The RP-TFO will also contain a region with reverse polarity to that of the polypurine region of the same. The 8aA,8aG-containing purine-rich region , the reverse polarity region, and the target pyrimidine-rich region together will form a pyrimidine-purine-pyrimidine triplex PNAS structure. See Fig. 33 A (1).

In one preferred embodiment of the present invention, the two regions of the RP-TFO have opposing polarity when connected at their 5' ends by a linker molecule. This assists triplex formation by preventing the formation of any hairpin structure. Hairpin folding of the RP-TFO itself, due to internal hydrogen bonding and duplex formation, will not be possible because the polypyrimidine region of the RP-TFO, while of identical sequence to the polypyrimidine target nucleic acid, is in the opposite orientation (polarity) to the complementary substituted-purine region. Thus, this structure is referred to as a "non-hairpin". The RP-TFO preferably has a length sufficient to allow specific binding to the target nucleic acid. The preferred embodiment will also have a number of 8aA and/or 8aG substituted nucleotides that will stabilize the PNAS at pH 7.0. Hoogsteen's bonds will form between the two parallel regions of the RP-TFO, while hydrogen bonds will form between the polypyrimidine target nucleic acid sequence, and the polypurine region of the RP-TFO. In combination, this will result in stable triplex formation at pH values greater than 5.5 (in the phisiological range).

In other preferred embodiments, ligands can be attached to the RP-TFO via spacers or linkers. This will allow the capture of a PNAS by its binding to a solid support coated with an appropriate affinity molecule having specificity for the ligand. An affinity molecule can be bound to the solid support by any means known to those with skill in the art, and can include, but is not limited to, a linker that attaches to a primary amine on the affinity molecule, and n-oxysuccinimide (NOS) coating the support surface.

In one preferred embodiment of the RP-TFO, the inverted orientation of the component regions results in two exposed 3' ends of the RP-TFO. In another preferred embodiment, the reversed regions may be linked via their 3' ends, so that the RP-TFO will have two exposed 5' ends. Hybridization of the RP-TFO is the first level of specificity of the assay.

Step IV: (Second level of Specificity) Initiate Signal Amplificaiton. For example, initiate MTRF formation. The MTRF complexes may be bound to the RP-TFO by the MTRF initiator probe or by the MTRF co-initiator probe. The example herein uses the co-initiator probe, though the MTRF -1, the MTRF initiator probe, can be substituted therefor.

Hybridize a reporter-MTRF co-initiator probe to the PNAS. The reporter-MTRF co-initiator probe has a section that complements a region in proximity to, or any position related thereof, the target nucleic acid sequence. This region of the probe will be of sufficient length to confer target sequence specificity. The reporter-MTRF co-initiator probe will also include polarity reversal within a linker, and a variable number of MTRF co-initiator sequences or polydA sequences separated by spacers. In one embodiment of the present invention, this probe is characterized as having two 5' ends. Other embodiments can have two 3' ends, or normal polarity. The probe can also include multiple MTRF co-initiator sequences, separated by spacer regions. In an alternative embodiment, the signal amplification system may be Duplex or Triplex Reporter technology, wherein the co-initiator probe includes polydA regions rather than the MTRF co-initiator sequences. See Fig. 33 A (2).

Step V: (Third Level of Specificity) Treat the sample with any single strand exonuclease capable of degrading single strand DNA and RNA in a 3'→5' direction. Non-target specific nucleic acid, unprotected by the PNAS, will be degraded. Excess RP-TFO molecules not bound to the target nucleic acid sequence will also be destroyed. The nuclease chosen must be unable to degrade the MTRF or reporter probes attached to the complex.

Step VI: The PNAS is absorbed to a solid substrate by means of a ligand attached to the RP-TFO, and an affinity molecule bound to the solid support. The choice of the ligand and the appropriate affinity molecule will be known to those skilled in the art.

Step VII: A wash step removes degraded nucleic acids, unbound or degraded RP-TFO probes, and enzymes.

Step VIII: (Fourth Level of Specificity) The MTRF-1 initiator probes are hybridized to complementary MTRF reporter/co-initiator probe sequences within the captured PNAS complex. In one embodiment, the MTRF-1 initiator probe has two 3' hydroxyl ends, one region complementary to the MTRF co-initiator sequence, a polarity reversal within a spacer, and another region with a 3' end that will complement a region within the MTRF nucleus structure. See Fig. 33 B (3).

Step IX. The PNAS/initiator probe complex is washed to remove unbound MTRF-1 initiator probe.

Step X: The bound PNAS-initiator probe complex is hybridized with MTRF nuclei. A low ratio of initiator probe to MTRF nuclei is used to hybridize each MTRF-1 initiator probe site with at least one MTRF nucleus. See Fig. 33 C (4).

Step XI: Hybridize the PNAS/MTRF nuclei saturated structure with a high ratio of MTRF nuclei to initiator probes, to obtain tight matrix formation, and attachment of a maximum number of MTRF labels to the PNAS complex, by self-aggregation of the MTRF nuclei. See Fig. 33 C (5).

Step XII: Wash to remove unbound MTRF nuclei.

Step XIII: Add a polydT probe containing multiple biotin moieties, and which will function in the MTRF, Duplex, and Triplex Reporter formats to increase the overall number of labels attached to the PNAS.

Step XIV: Wash to remove excess unbound probe

Step XV: Add a streptavidin-enzyme, dye label, or other label conjugate known to those skilled in the art.

Step XVI: Add a substrate for the label enzyme to react with and generate any signal, or directly measure the dye or other label attached to the conjugate by any means known to those skilled in the art.

The numerical designations in Figures 33A-33C herein are represented by:

1, is single strand nucleic acid containing a polypyrimidine target region.

2, is the Reverse Polarity-Triplex Forming Oligonucleotide (RP-TFO)

3, is the polypyrimidine 3' end of the RP-TFO

4, is the polypurine 3' end of the RP-TFO substituted with 8aA and/or 8aG.

5, are modified bases 8- aminoadenosine (8aA) and 8- aminoguanosine (8aG).

6, is the polypyrimidine target nucleic acid sequence.

7, are Hoogsteen's bonds between the polypyrimidine region of the RP-TFO and the polypurine region of the RP-TFO.

8, is the hydrogen bonding formed between the target polypyrimidine region and the polypurine region of the RP-TFO.

9, is a linker connecting the two regions of reverse polarity of the RP-TFO.

10, is a primary amine binding to a n-oxysuccinimide coated solid support

11, is the PNAS and Triplex complex.

12, is the reporter MTRF-co-initiator Probe

13, is the region complementary to a region in proximity to the target.

14, is a spacer

15, are the GC rich co-initiator sites that have a region complementary to the MTRF-1 initiator probe.

16, is the reversal of polarity internally within the MTRF reporter/co-initiator.

17, is the MTRF-1 initiator probe.

18, is the GC-rich region of the MTRF-1 complementary to the co-initiator sequences on the PNAS.

19, is a spacer

20, is the GC-rich region of the MTRF-1 complementary to a region on the MTRF nucleus.

21, is the MTRF nucleus.

22, are the polydA regions of the MTRF nucleus structure acting as a core for the Duplex and Triplex Reporter techniques.

23, are the biotinylated polydT signal amplification probes with an affinity molecule or dye attached.

24, is the label; enzyme, dye, or other molecule.

25, is the GC-rich region of the MTRF nucleus that binds to the initiator probe, and other MTRF nuclei.

It should be understood, of course, that the foregoing relates only to preferred embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the invention as set forth in the appended claims.

Claims

What is claimed is

1. A Multiple Triplex Reporter Forming (MRTF) self-complexing probe composition comprising:

an initiator probe;

at least one first MRTF probe and at least one second MRTF probe, wherein first and second MRTF probes are complexed with the initiator probe to form triplex nucleic acid structures;

a plurality of triplex nucleic acid structures that together form the self-complexing probe.

2. A method for the amplification of a nucleic acid hybridization signal by the formation of a first Multiple Triplex Reporter Formation (MRTF) complex, comprising the steps of:

isolating a biological sample containing an antigen

adding to the biological sample an antibody/co-initiator complex

forming a second complex between the antigen in the biological sample and the antibody/co-initiator;

capturing the second complex by attaching it to a solid support;

hybridizing the captured second complex with a MRTF-1 initiator probe;

hybridizing the captured second complex with a low number of MTRF nucleii;

hybridizing the captured second complex with a high number of MRTF nucleii to form a third complex;

hybridizing the thrid complex with a multi-biotinylated poly dT oligonucleotide

adding label-strptavidin conjgate

adding a subtrate for the label.

3. The method of claim 2, wherein the nucleic acid is selected from either DNA or RNA.

4. The method of claim 3, wherein the DNA is single-stranded.

5. The method of claim 3, wherein the DNA is double-stranded.

6. A method for cleavage of a nucleic acid target comprising the steps of:

isolating nucleic acid containing a target sequence, from a biological sample;

denaturing the nucleic acid to single strand form;

hybridizing the denatured nucleic acid strands with cutter probes to form a nucleic acid duplex, wherein the cutter probes include 5-bromouracil

generating free radicals within the nucleic acid duplex and thereby cleaving the duplex nucleic acid, and

isolating the cleaved nucleic acid target sequence.

7. The method of claim 6, wherein the nucleic acid is selected from either DNA or RNA.

8. The method of claim 7, wherein the DNA is single-stranded.

9. The method of claim 7, wherein the DNA is double-stranded.

10. An antibody-MTRF complex, comprising:

(a) an antibody capable of binding to the target antigen;

(b) a GC-rich MTRF co-initiator probe linked to the second antibody; and

(c) at least one MTRF nucleus attached to the co-initiator probe to provide a means of labeling the antibody-MTRF complex.

11. An antibody-MTRF complex, comprising:

(a) a target antigen;

(b) a first antibody capable of binding to the target antigen;

(c) a second antibody capable of binding to the first antibody, wherein the first and second antibodies are raised in different animal species;

(d) a GC-rich MTRF co-initiator probe linked to the second antibody; and

(e) at least one MTRF nucleus attached to the co-initiator probe to provide a means of labeling the antibody-MTRF complex.

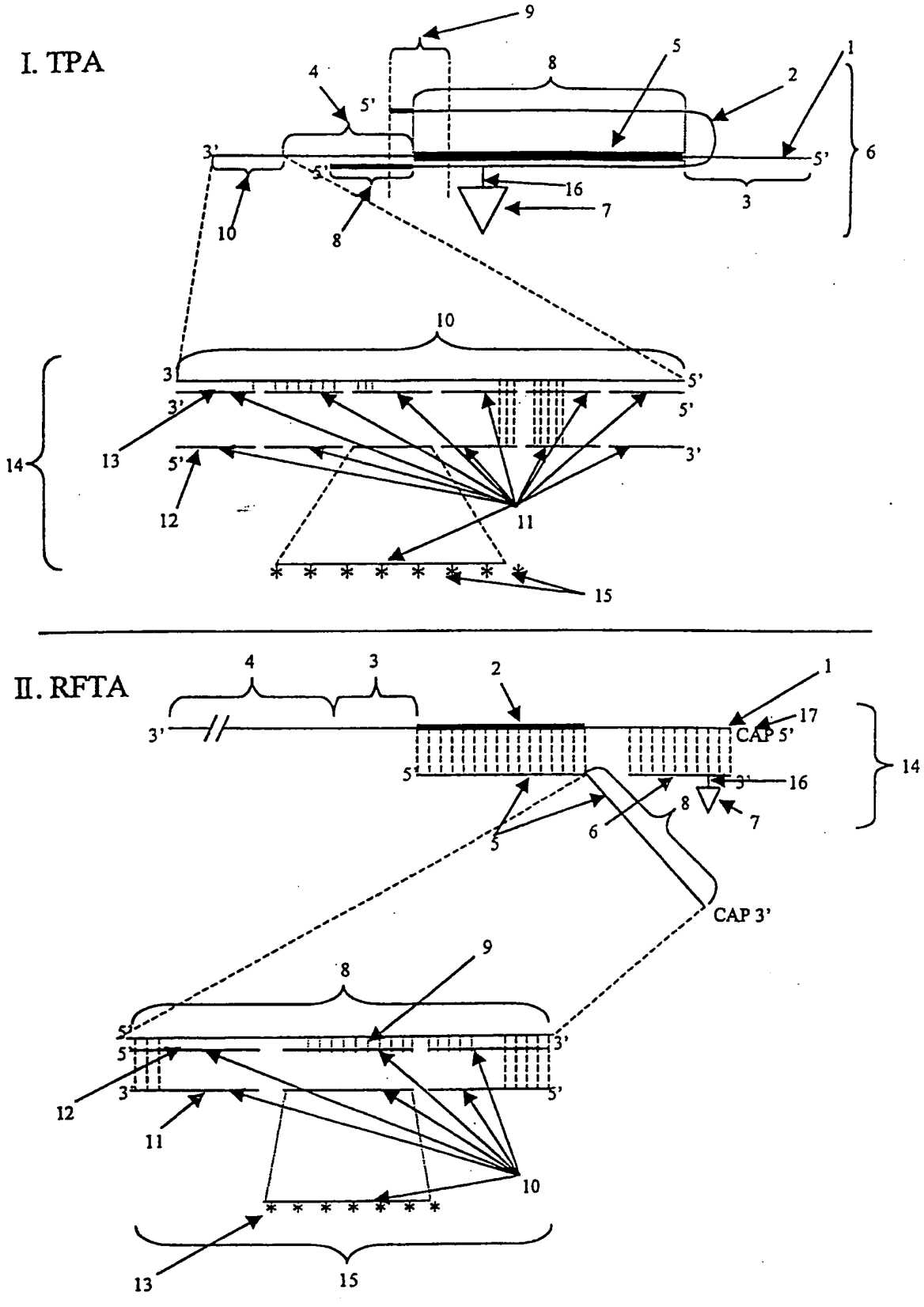
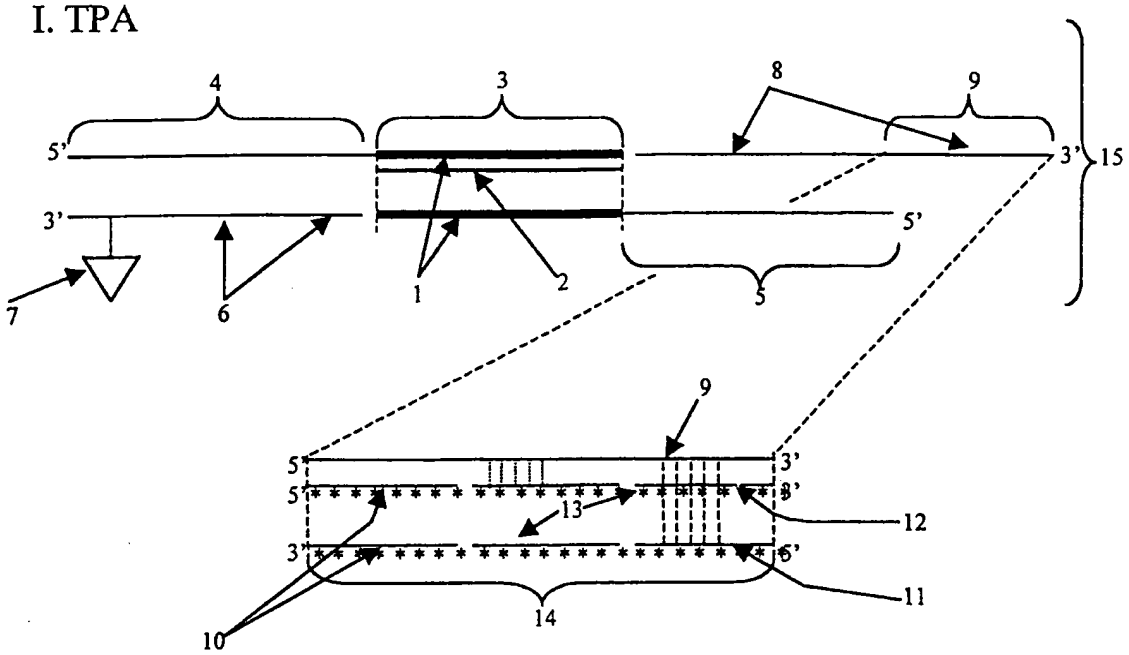


Fig. 1

I. TPA



II. RFTA

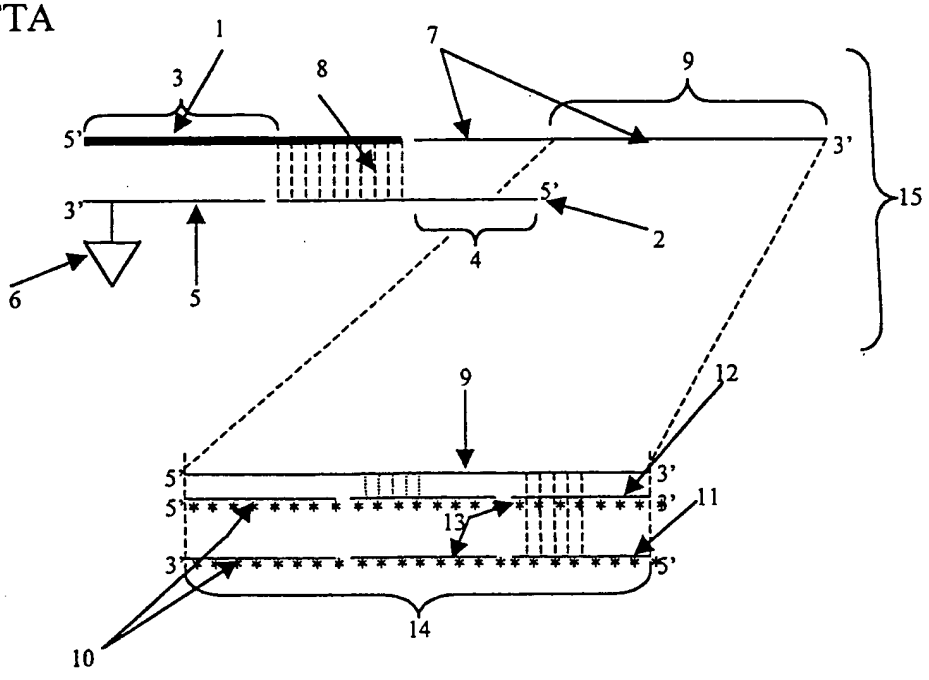


Fig. 2

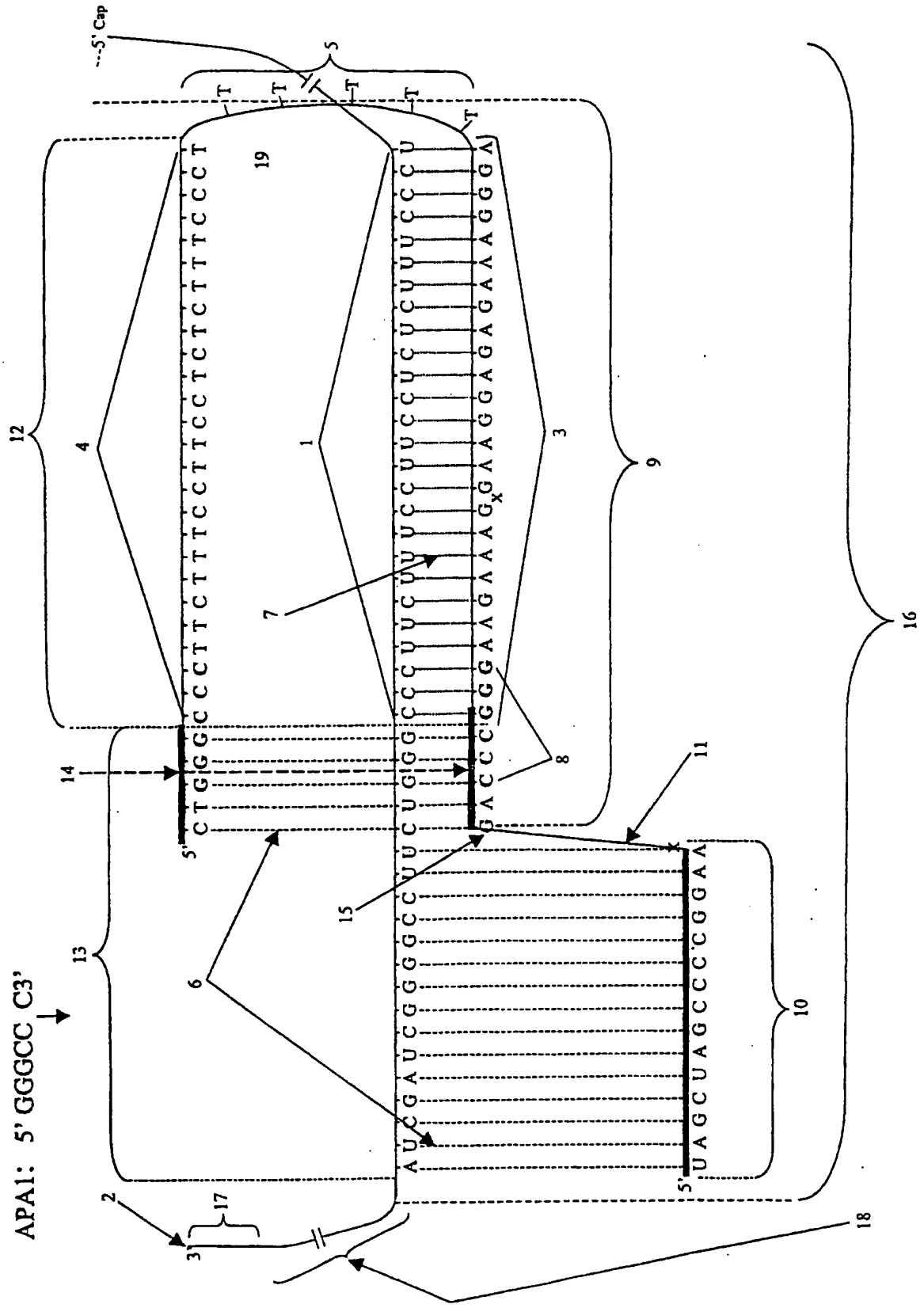


Fig. 3

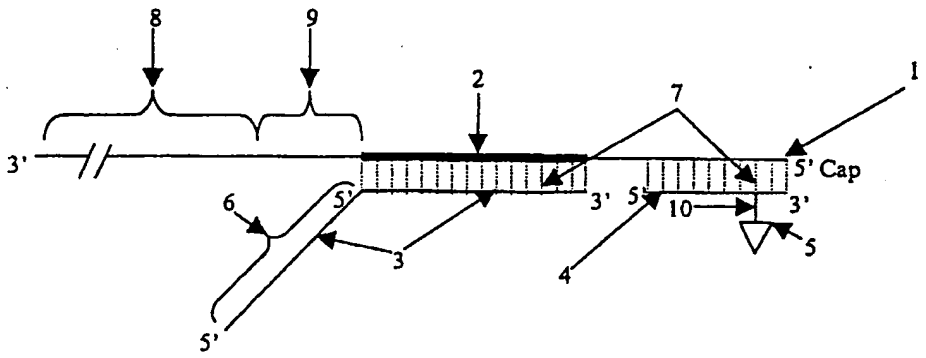


Fig. 4

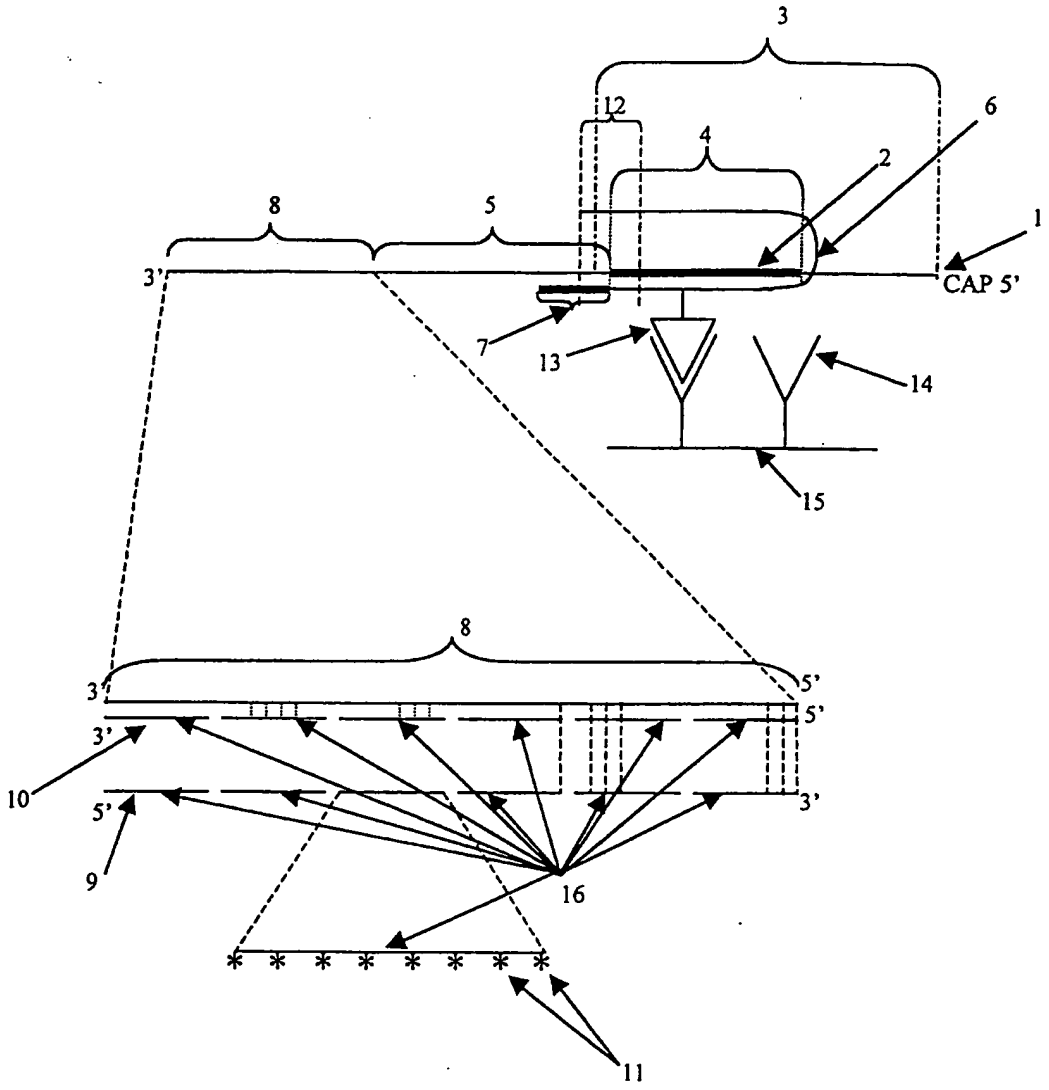


Fig. 5

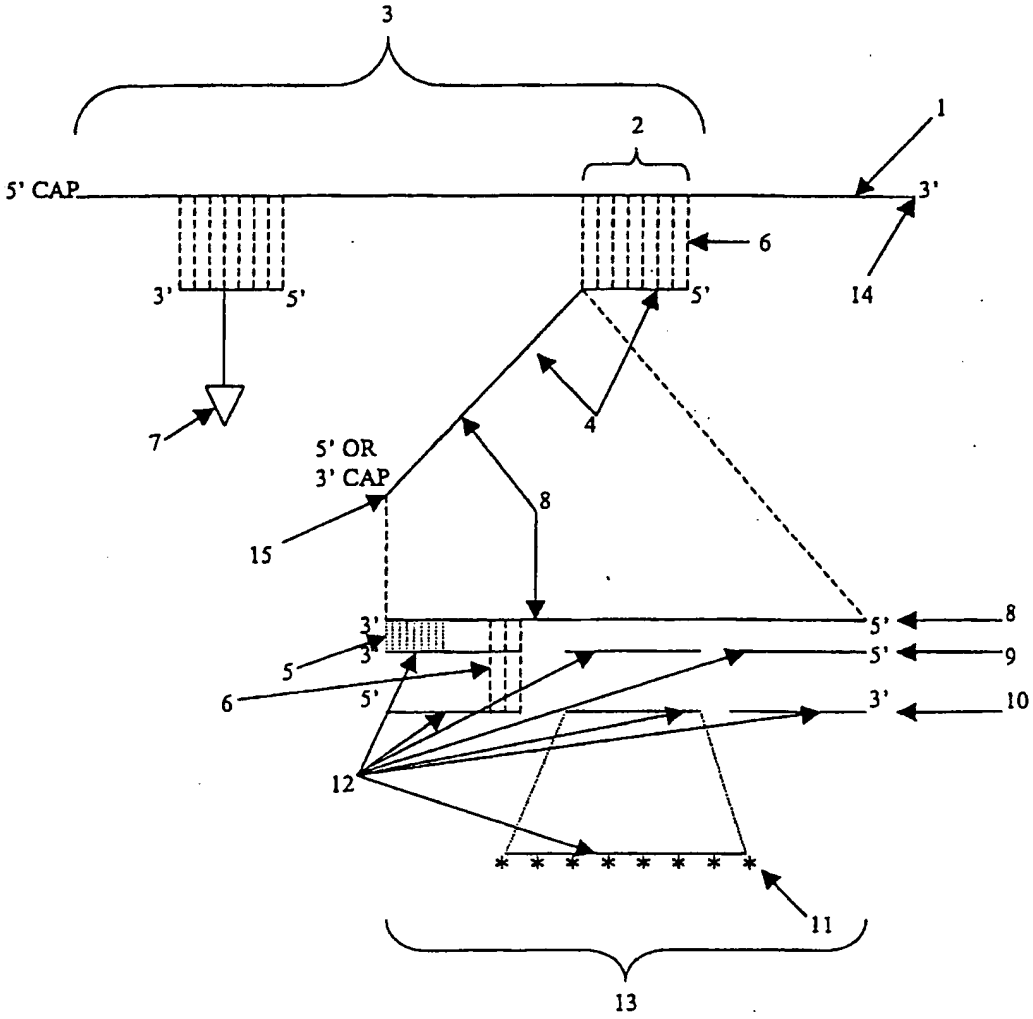
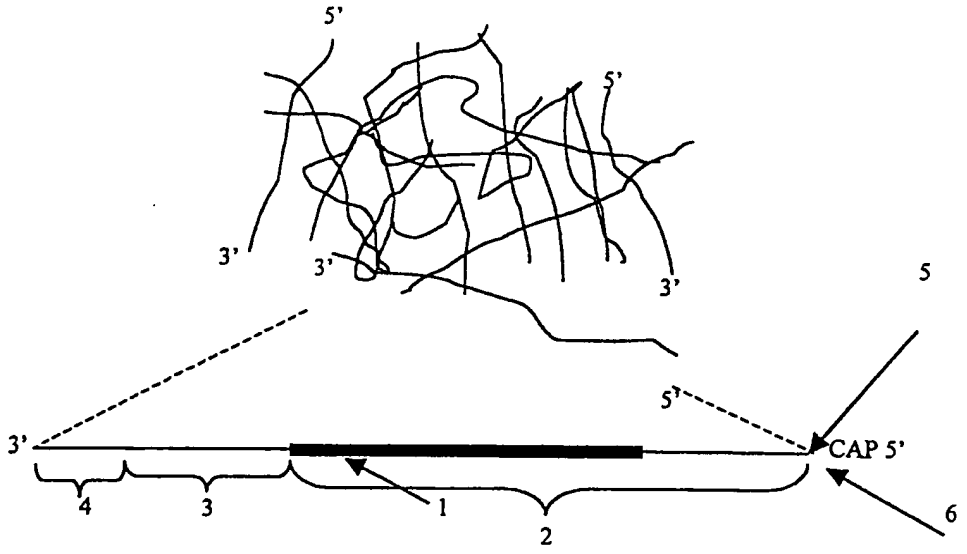
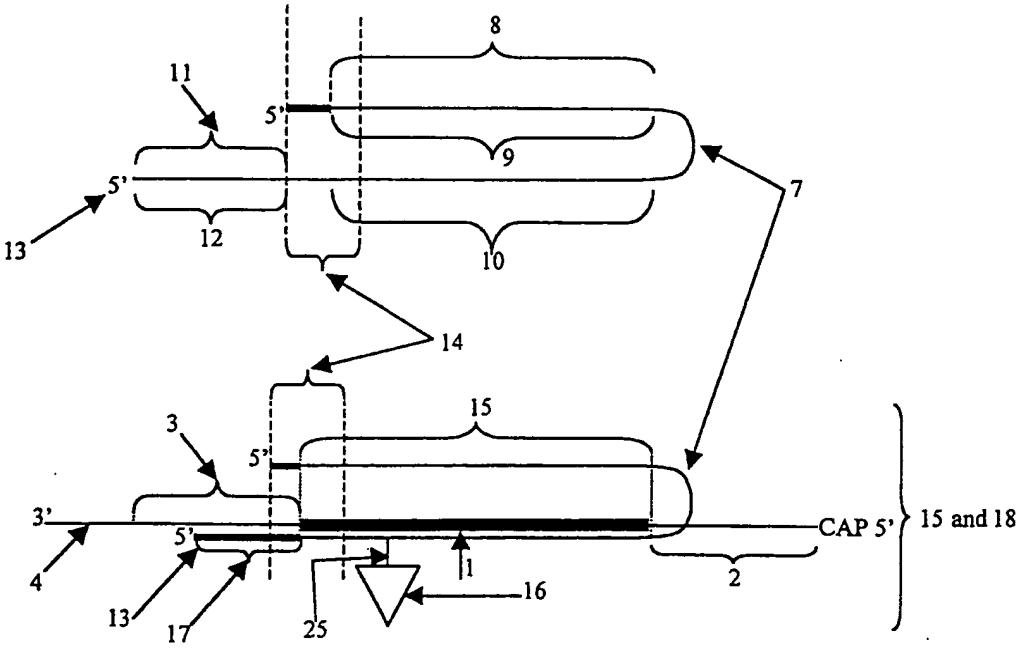


Fig. 6

1.


5'
5'
3'
3'
3'
5
5'
CAP 5'
3'
4
3
1
2
6

2.


8
11
5'
9
5'
7
13
12
10
14
15
3
5'
3'
CAP 5'
15 and 18
5'
4
1
2
13
16
17
25

3.

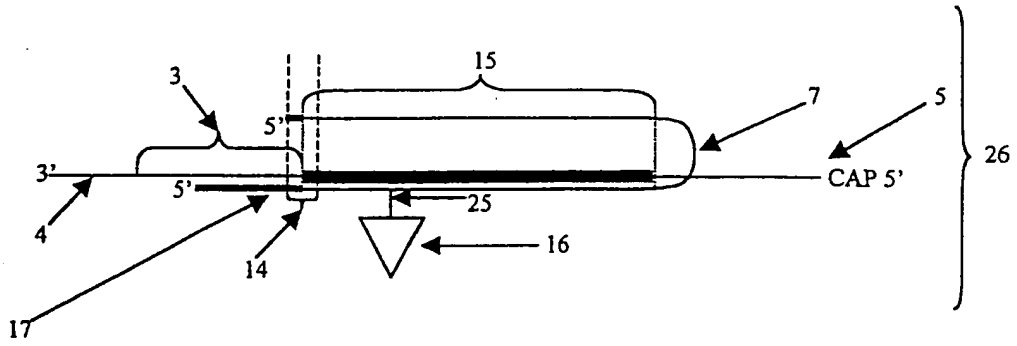
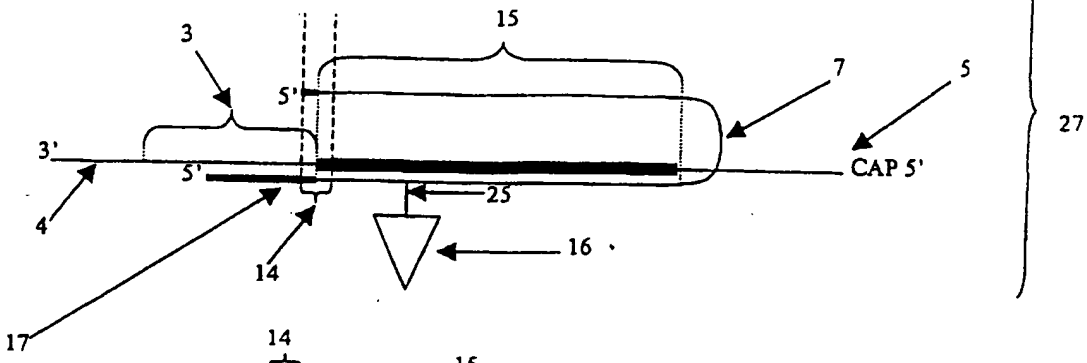
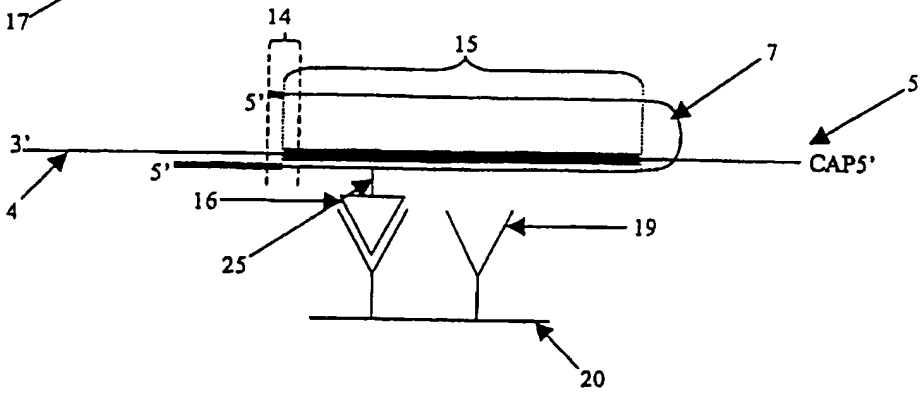

15
3
7
5
5'
3'
CAP 5'
26
5'
25
4
16
14
17

Fig. 7A

4.


3
15
5'
7
5
3'
5'
CAP 5'
25
4
16
14
17
27

5.


14
15
7
5'
5
3'
5'
CAP5'
4
16
19
25
20

6.

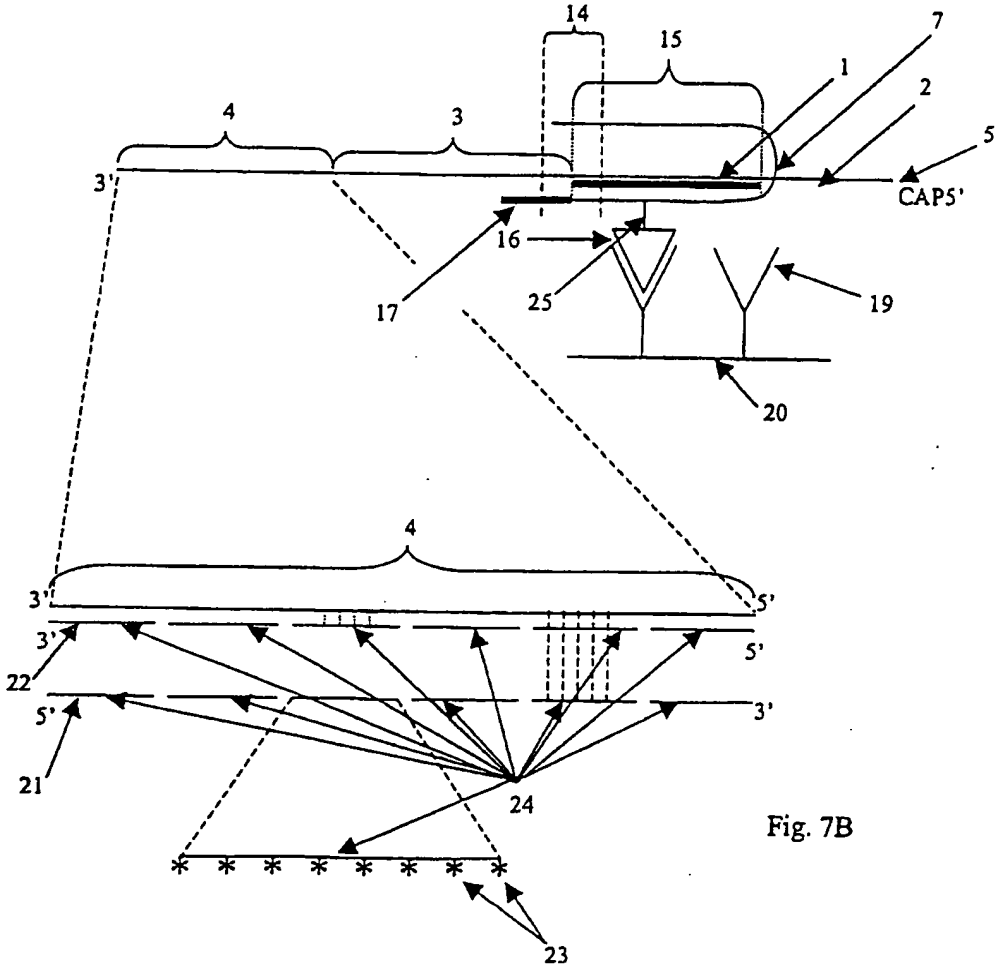
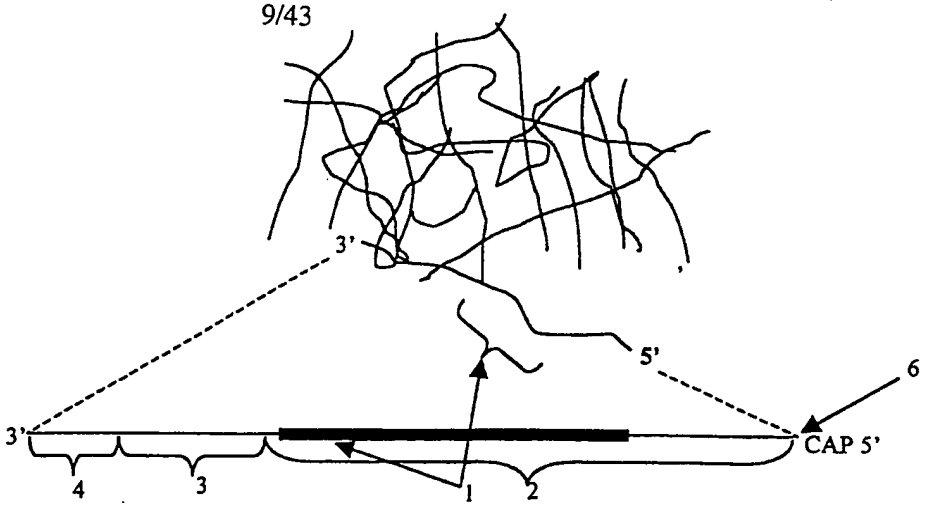
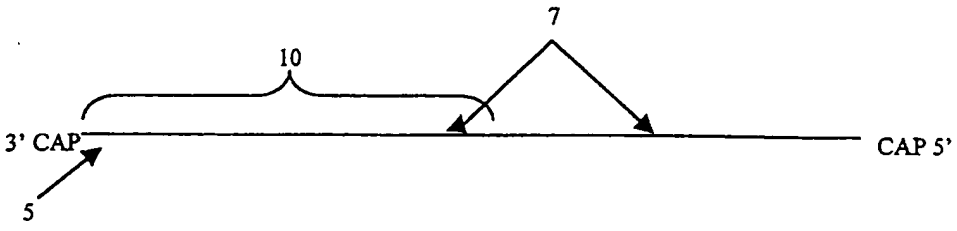

14
15
7
1
2
4
3
5
3'
CAP5'
16
17
25
19
20
4
3'
5'
3'
5'
22
5'
3'
21
24
23

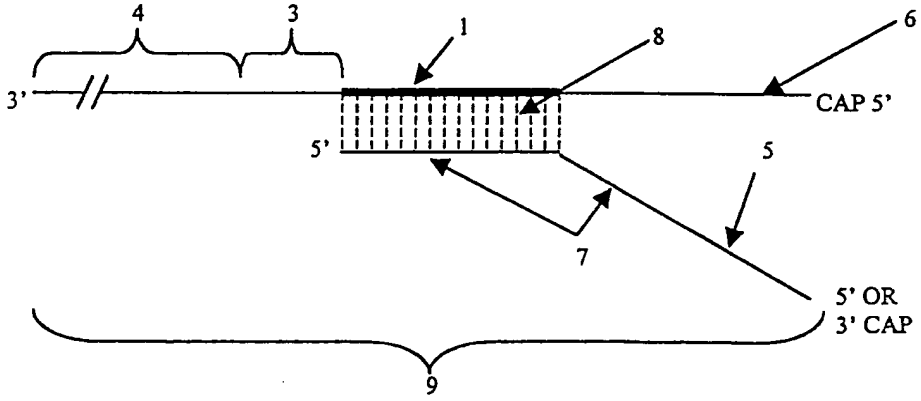
Fig. 7B

1.


3'
5'
6
3'
CAP 5'
4
3
1
2

2.


7
10
3' CAP
CAP 5'
5


4
3
1
8
6
3'
CAP 5'
5'
5
7
5' OR
3' CAP
9

3.

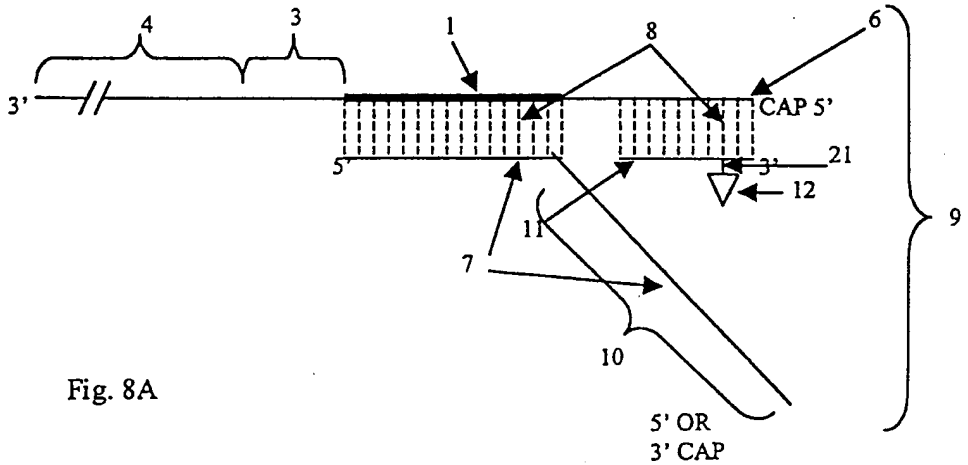
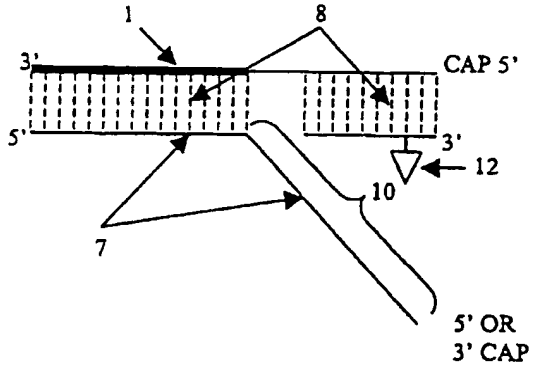
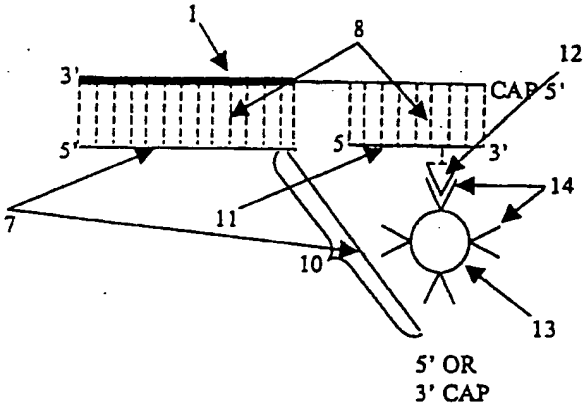

4
3
1
8
6
3'
CAP 5'
5'
3'
21
12
11
9
7
10
5' OR
3' CAP

Fig. 8A

4.


1
8
3'
CAP 5'
5'
3'
12
10
7
5' OR
3' CAP

5.


1
8
12
3'
CAP 5'
5'
5'
3'
14
11
7
10
13
5' OR
3' CAP

6.

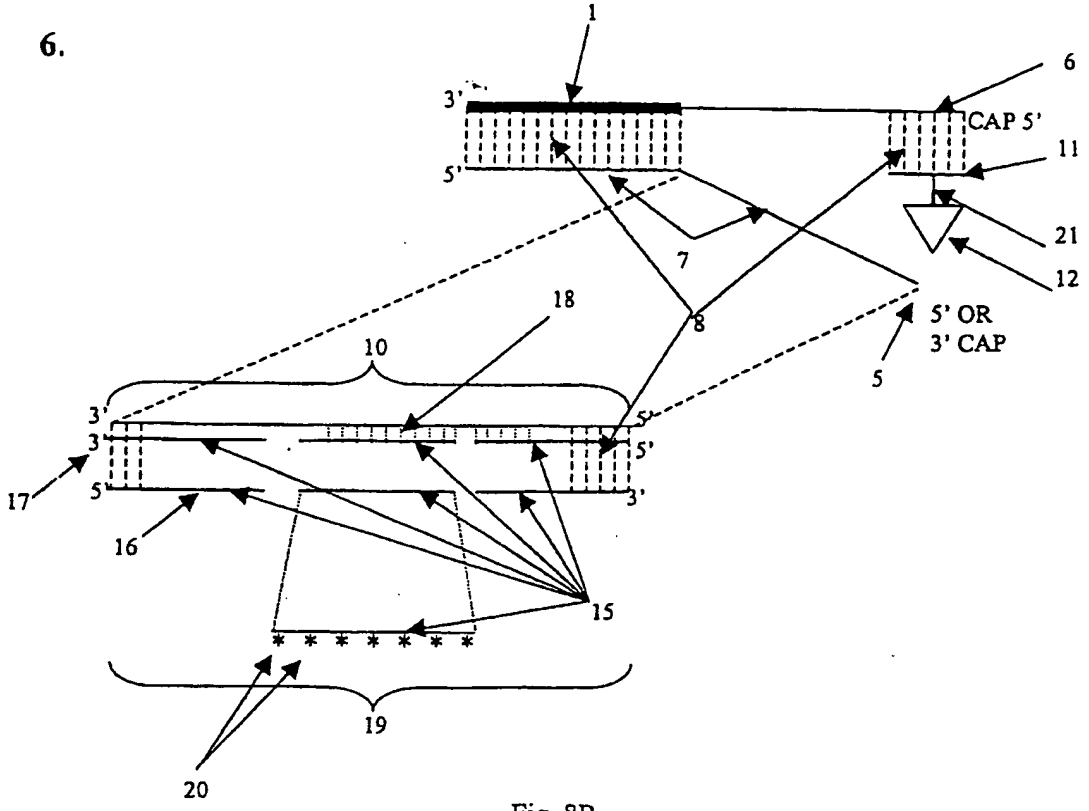
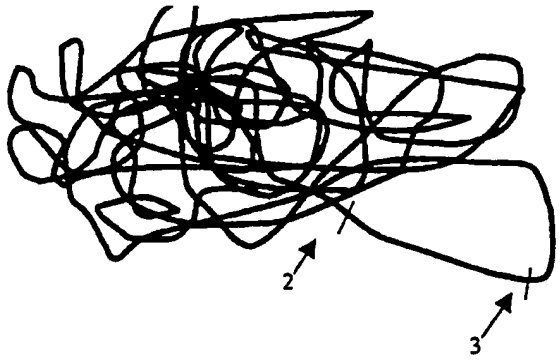
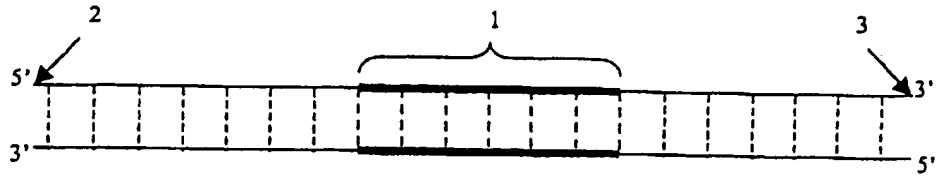

1
6
3'
CAP 5'
5'
11
21
7
12
18
8
5' OR
3' CAP
10
5
3'
5'
3'
5'
17
5'
3'
16
15
19
20

Fig. 8B

1.



2.



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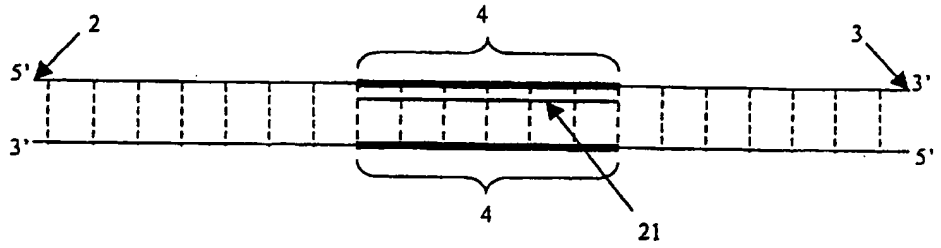
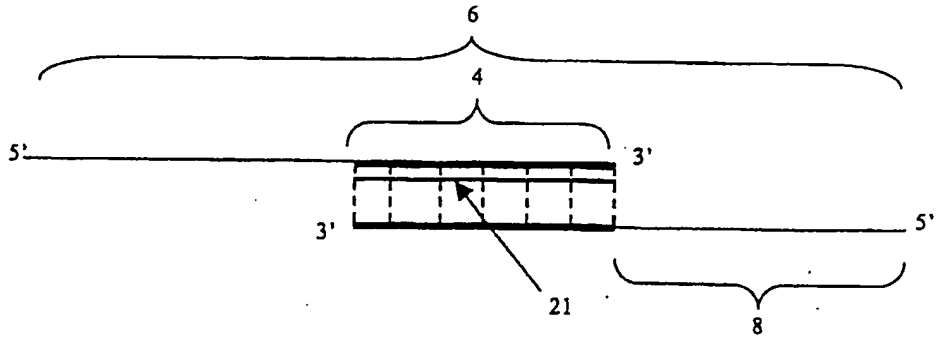


4.



5.

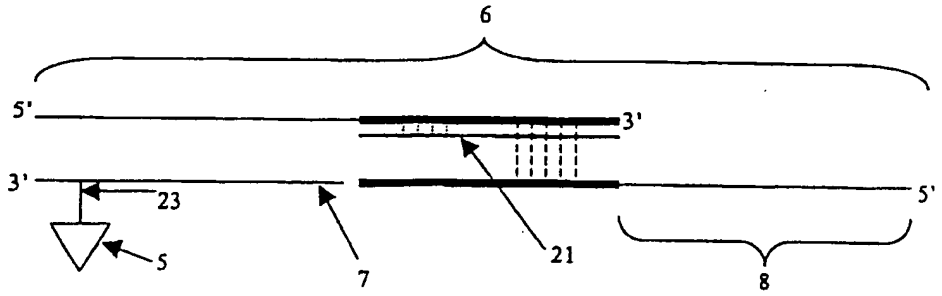
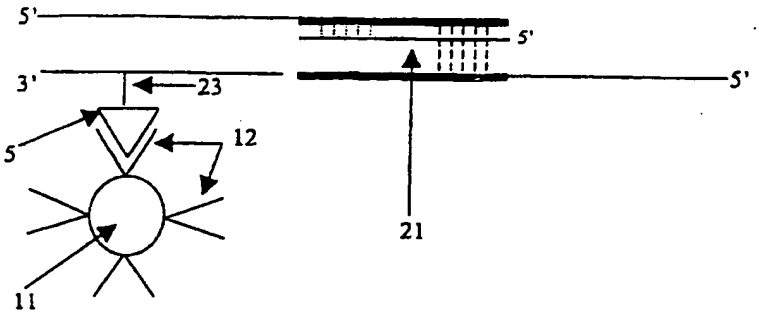
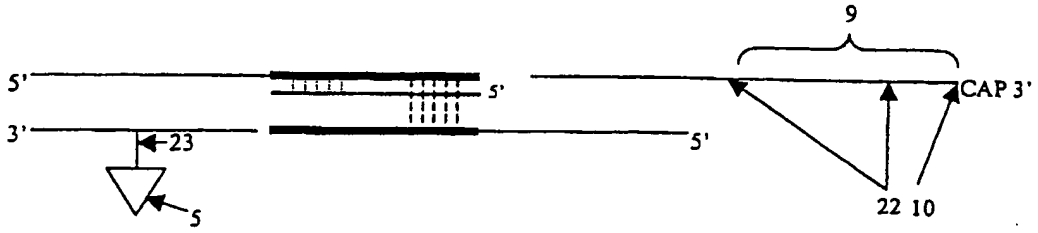


Fig. 9A

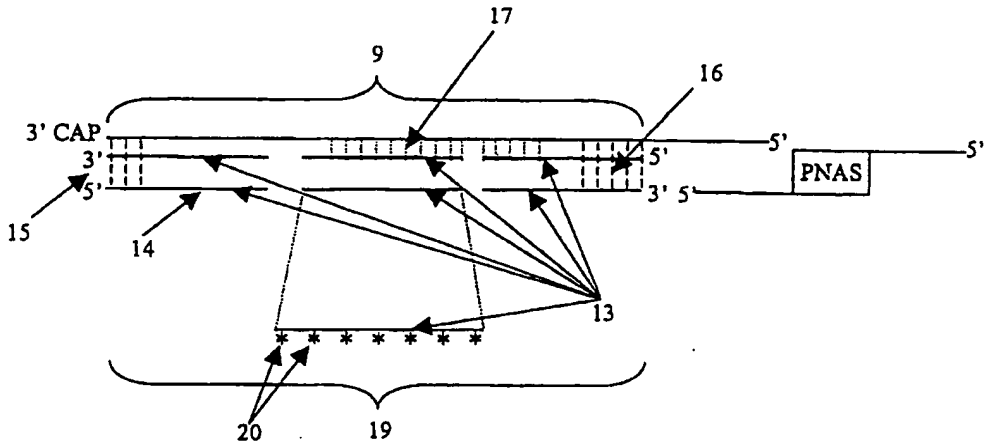
6.


5'
5'
3'
23
5'
5'
12
21
11

7.


9
5'
5'
CAP 3'
3'
23
5'
5
22
10

8.


17
9
16
3' CAP
5'
5'
3'
5'
3'
5'
PNAS
5'
15
14
13
20
19

9.

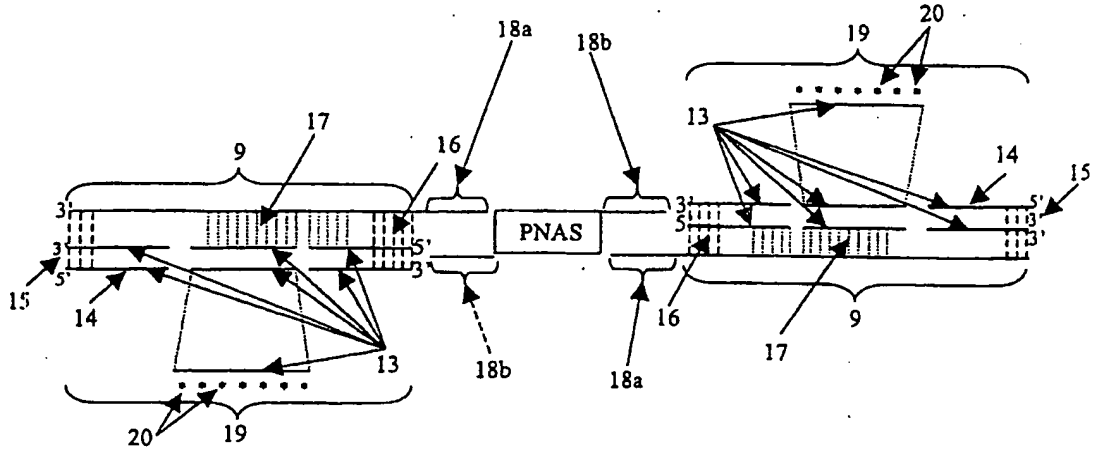

18a
18b
19
20
17
9
16
13
14
15
PNAS
15
14
13
18b
18a
16
17
9
20
19

Fig. 9B

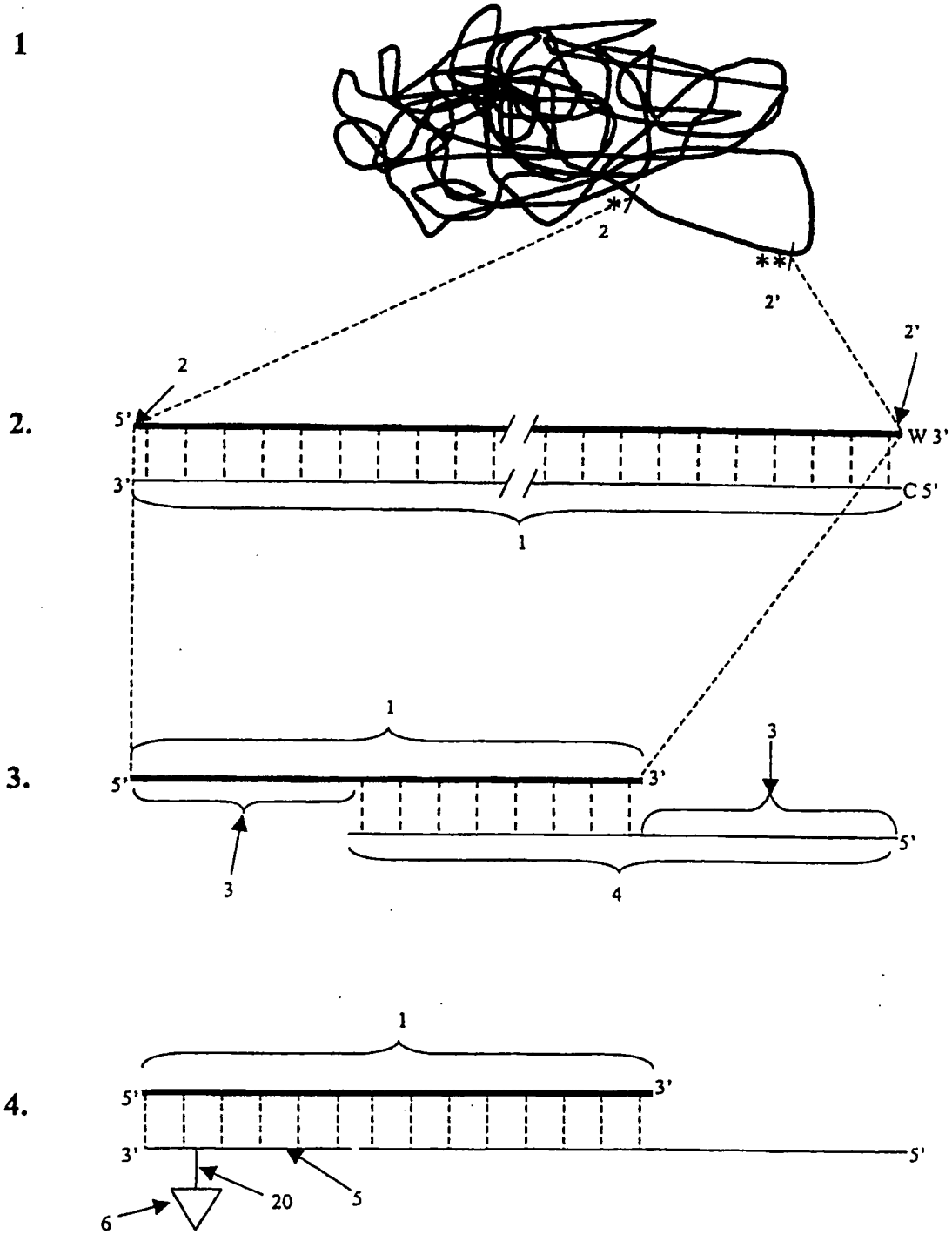
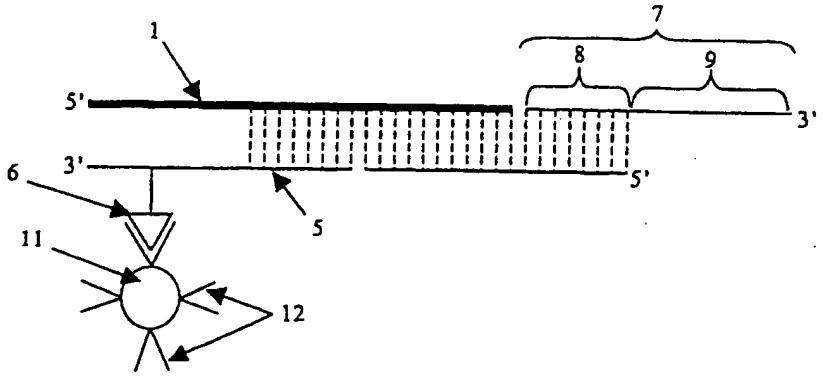
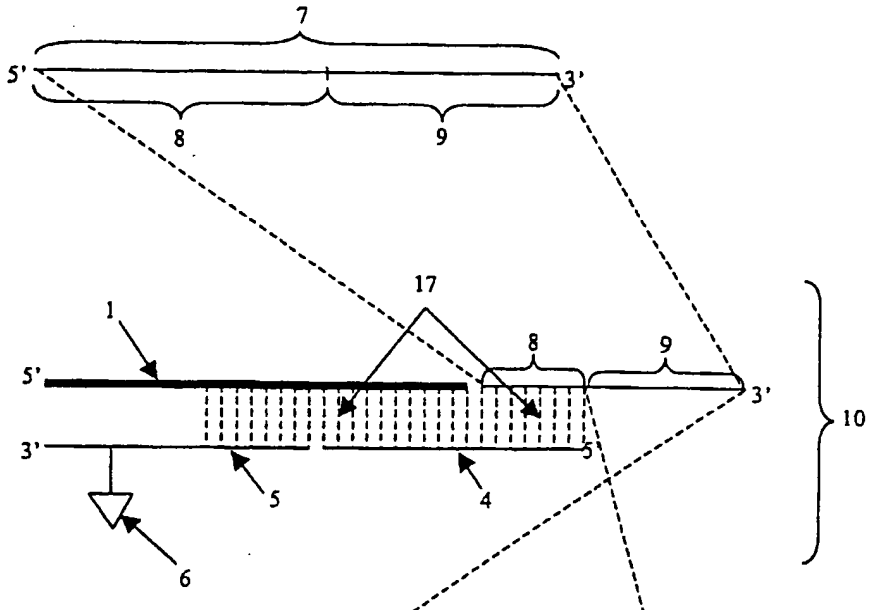


Fig. 10A

5.


1
7
8
9
5'
3'
3'
5'
6
5
11
12

6.


7
5'
3'
8
9
17
1
8
9
5'
3'
10
3'
5
5
4
6

7.

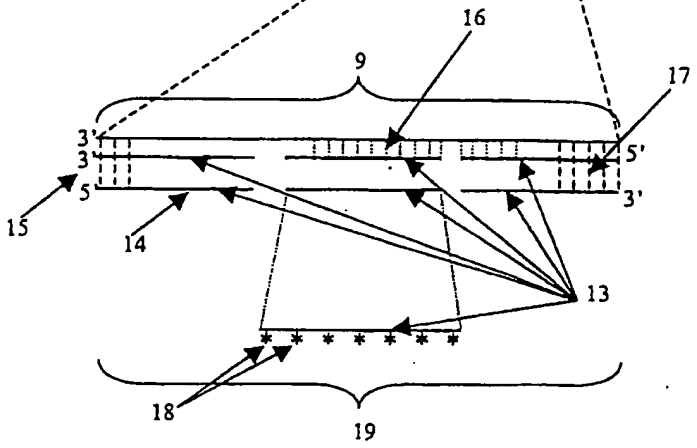
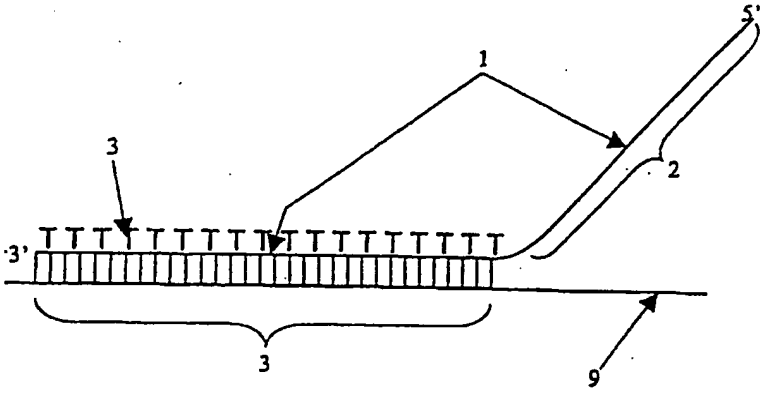
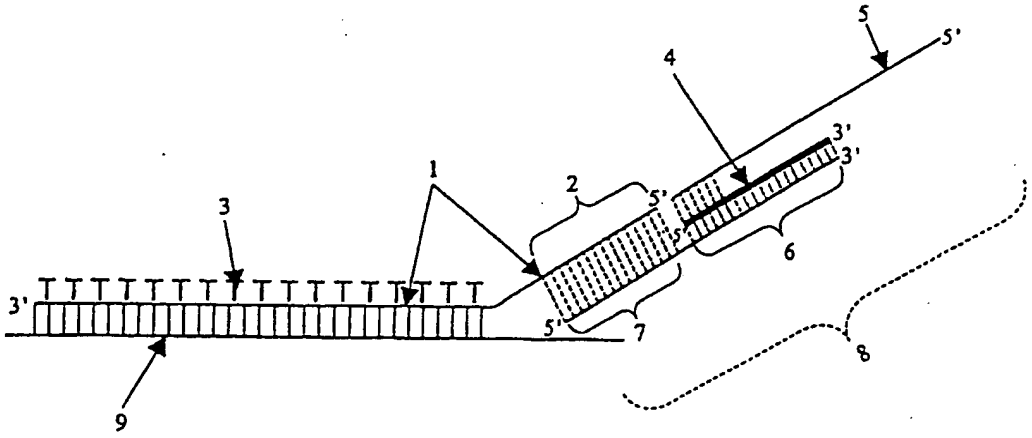

16
9
17
3'
3'
5'
5
3'
15
14
13
18
19

Fig. 10B

1.



2.



3.

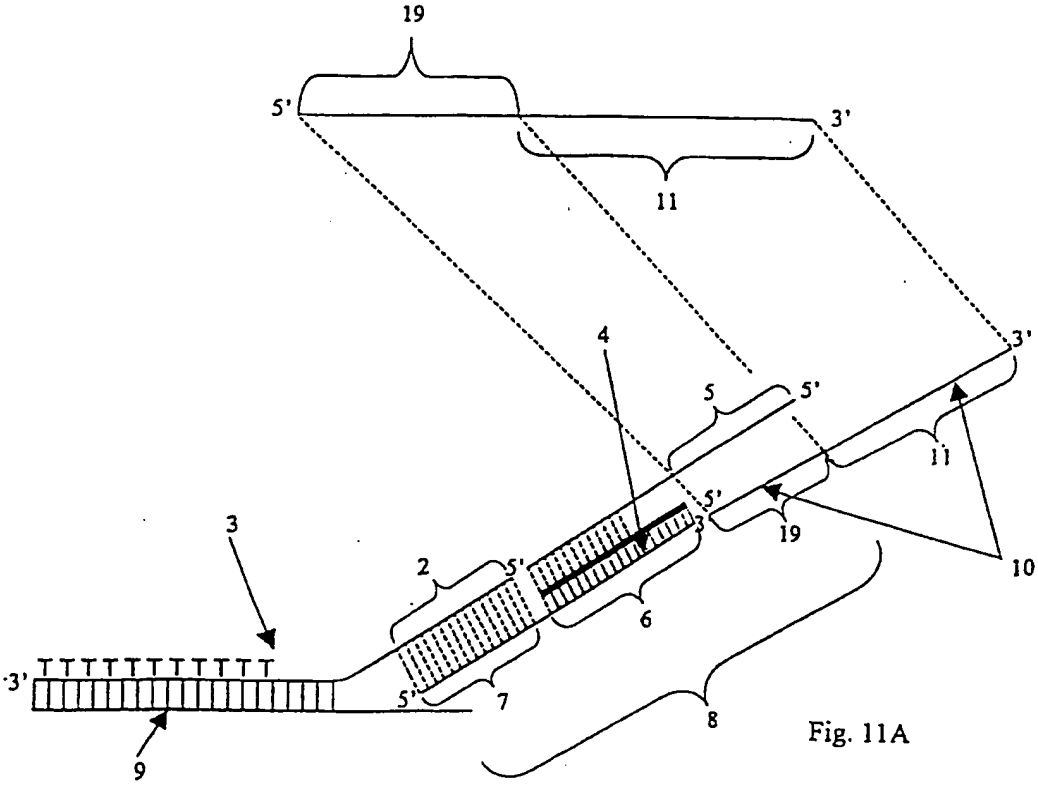
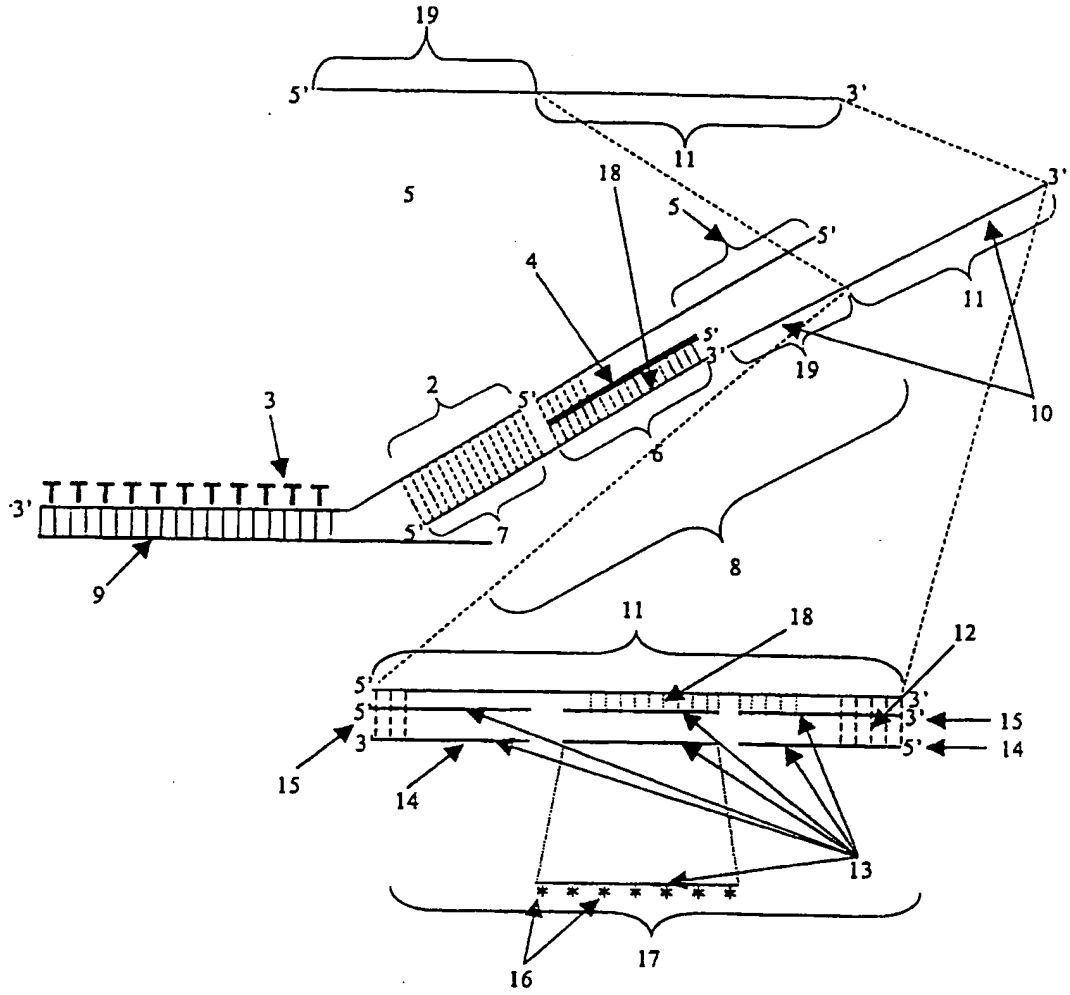


Fig. 11A

4.


19
5'
3'
11
5
18
5
5'
4
11
3'
19
10
3
2
5'
6
3'
5'
7
9
8
11
18
12
5'
3'
5'
3'
15
3
5'
14
15
14
13
16
17

5.

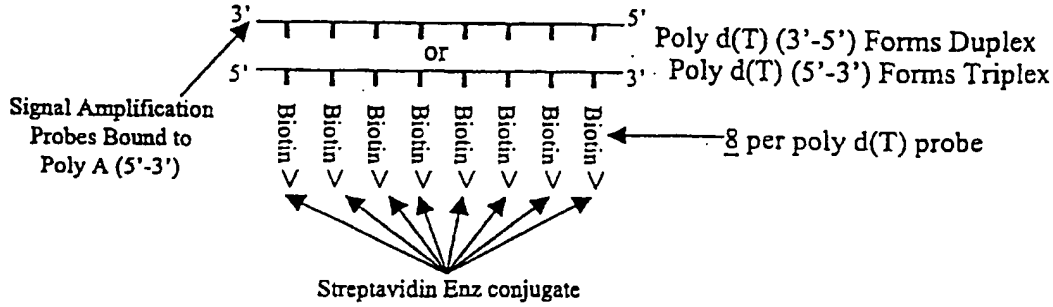

3'
5'
Poly d(T) (3'-5') Forms Duplex
or
5'
3'
Poly d(T) (5'-3') Forms Triplex
Signal Amplification Probes Bound to Poly A (5'-3')
Biotin
Biotin
Biotin
Biotin
Biotin
Biotin
Biotin
Biotin
8 per poly d(T) probe
Streptavidin Enz conjugate

Fig. 11B

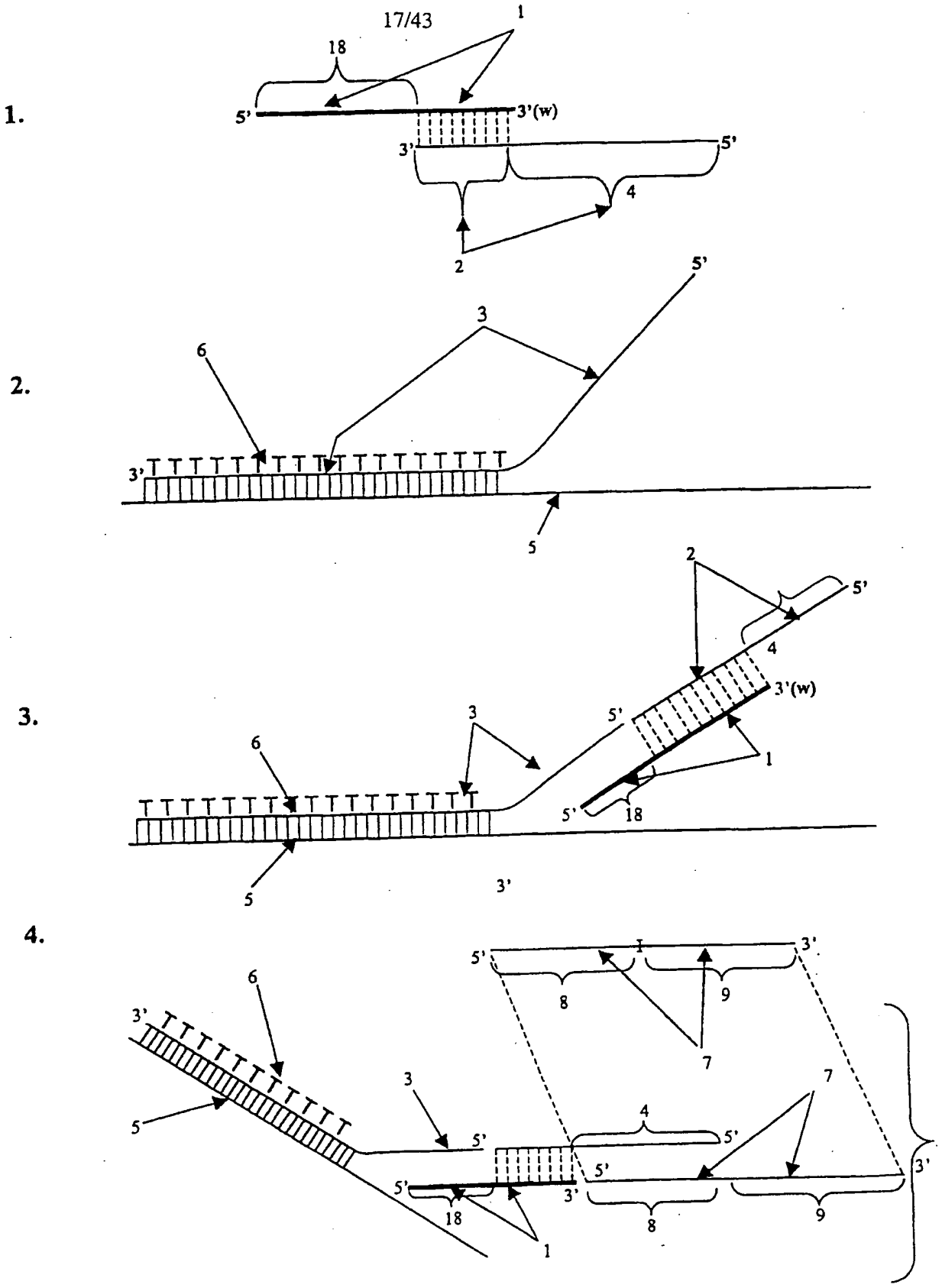

1.
18
1
5'
3'(w)
3'
5'
4
2
2.
5'
3
6
3'
5
3.
2
5'
4
3'(w)
3
5'
6
1
5'
18
5
3'
4.
5'
3'
8
9
7
6
3'
3
7
5
5'
4
5'
5'
5'
3'
3'
18
1
8
9

Fig. 12A

5.

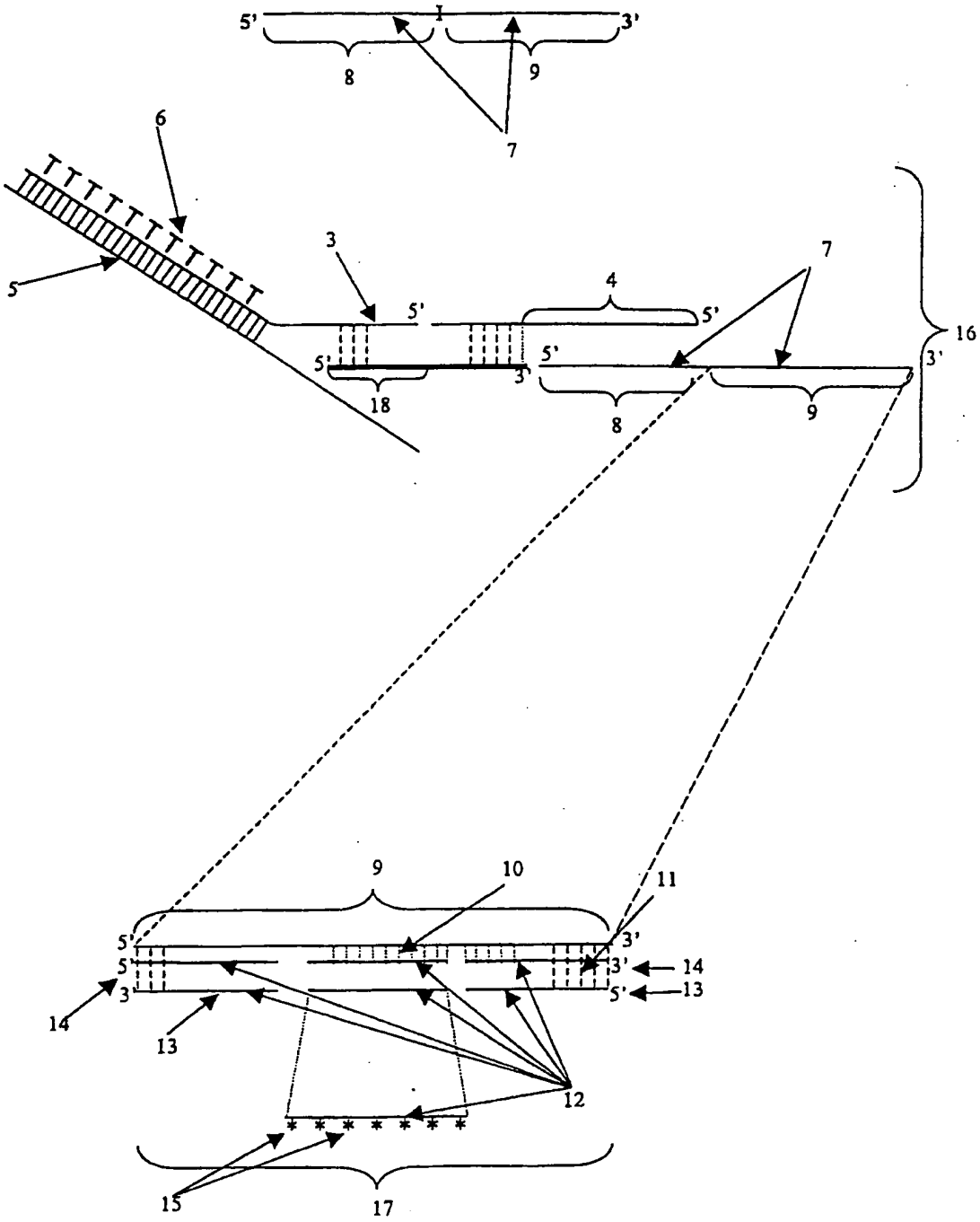
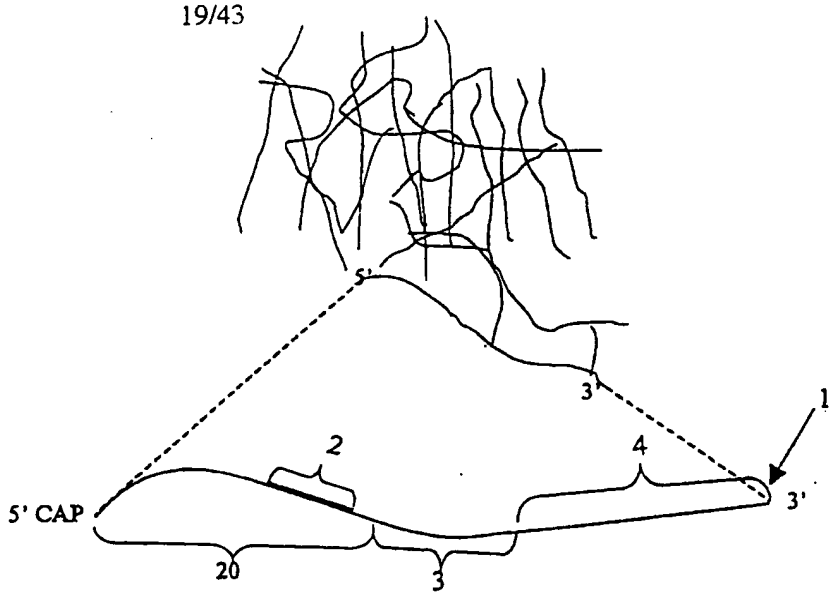
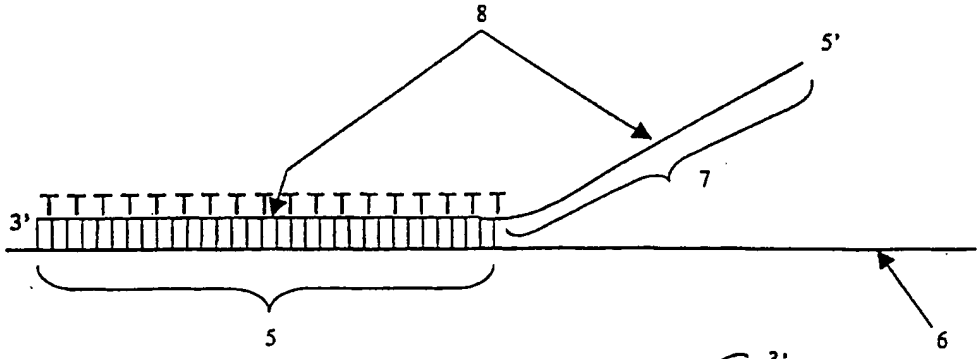


Fig. 12B

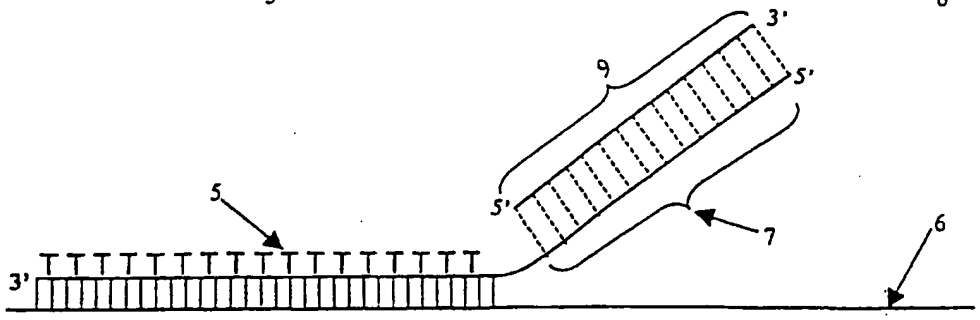
1.



2.



3.



4.

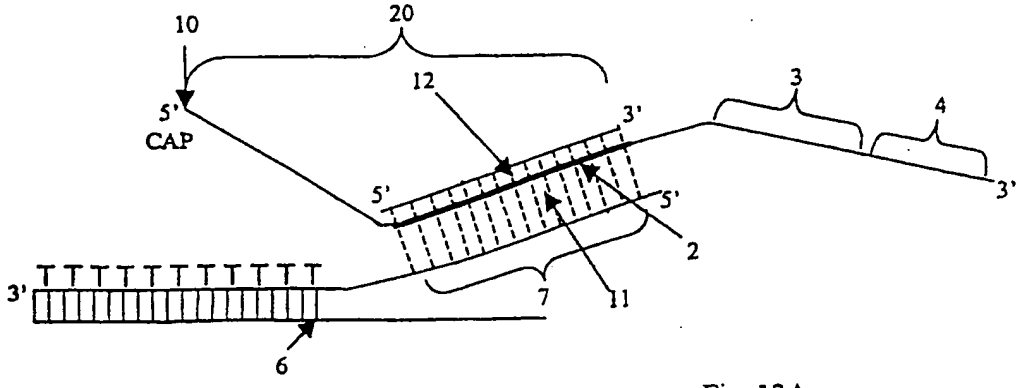
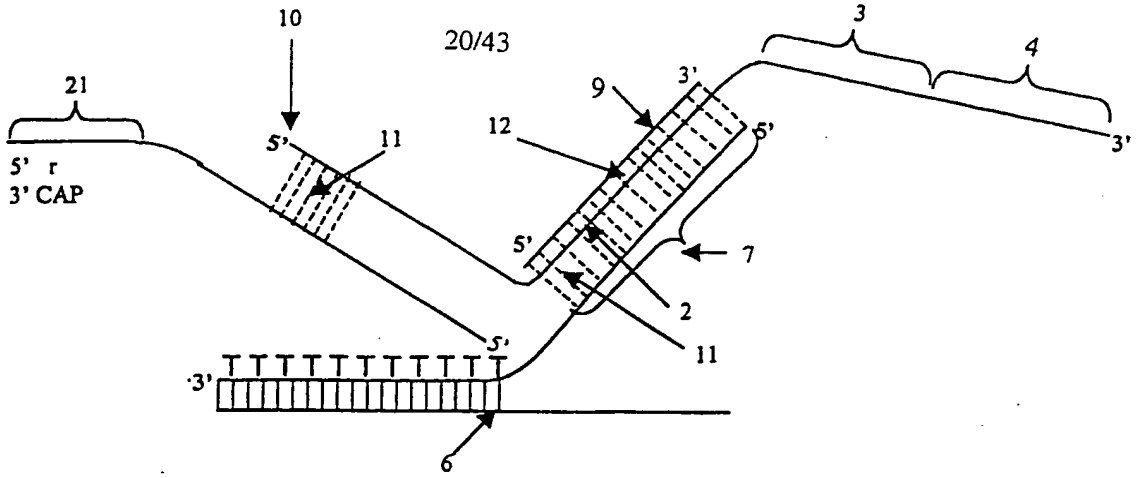
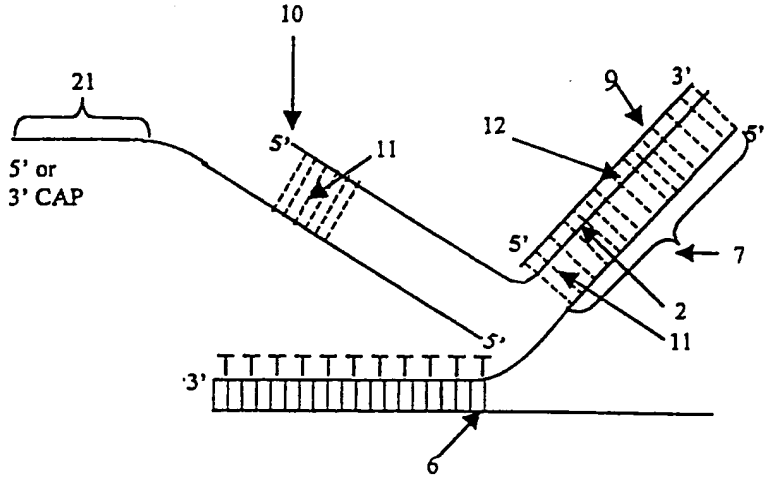


Fig. 13A

5.


10
21
3
4
9
3'
12
5'
11
5' r
3' CAP
7
2
11
3'
6

6.


10
21
9
3'
12
5'
11
5' or
3' CAP
7
2
11
3'
6

7.

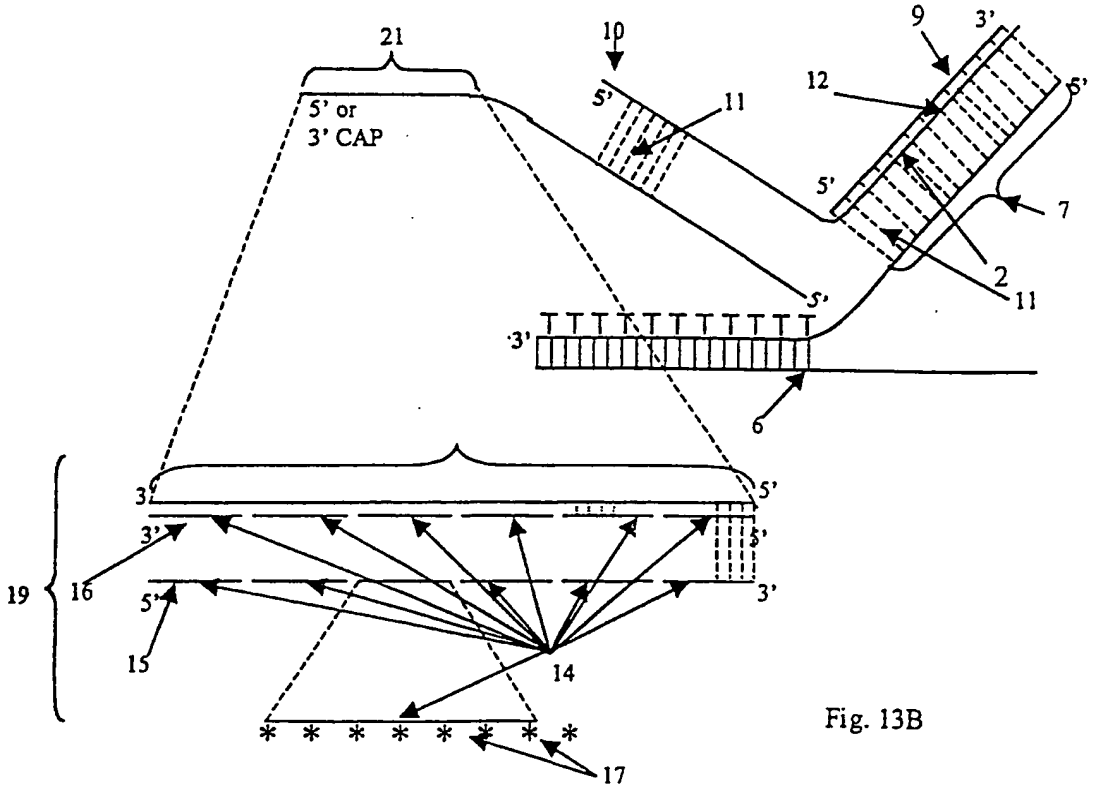
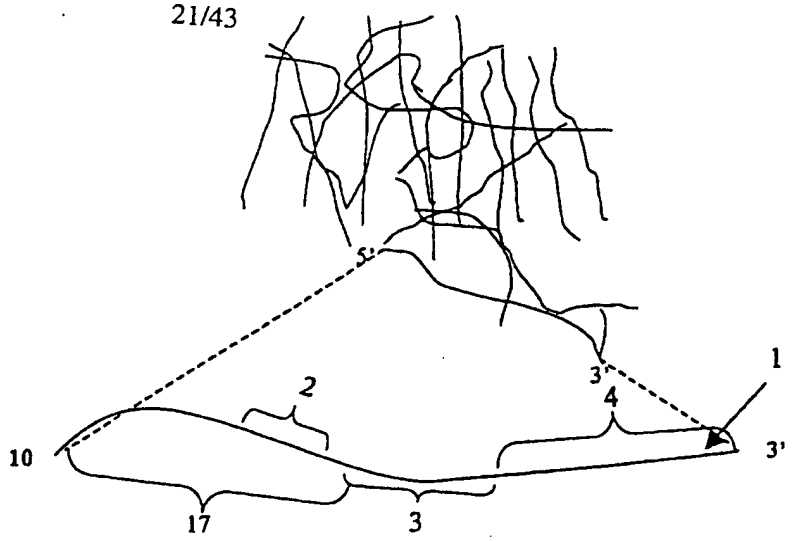
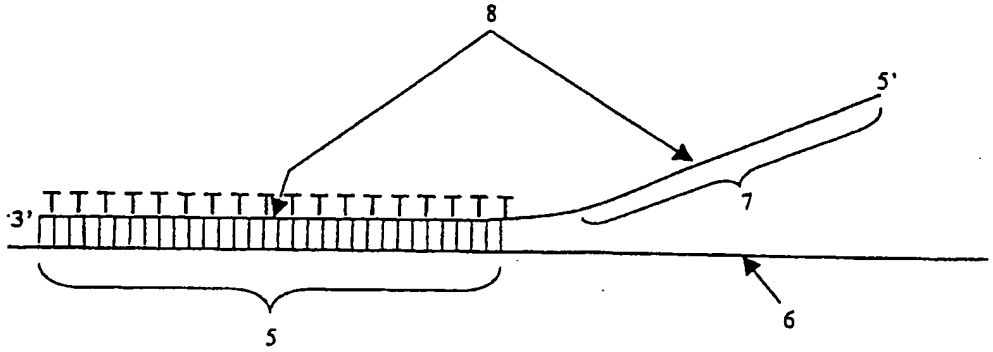

21
10
9
3'
5' or
3' CAP
12
5'
11
7
2
11
3'
6
19
16
15
14
17

Fig. 13B

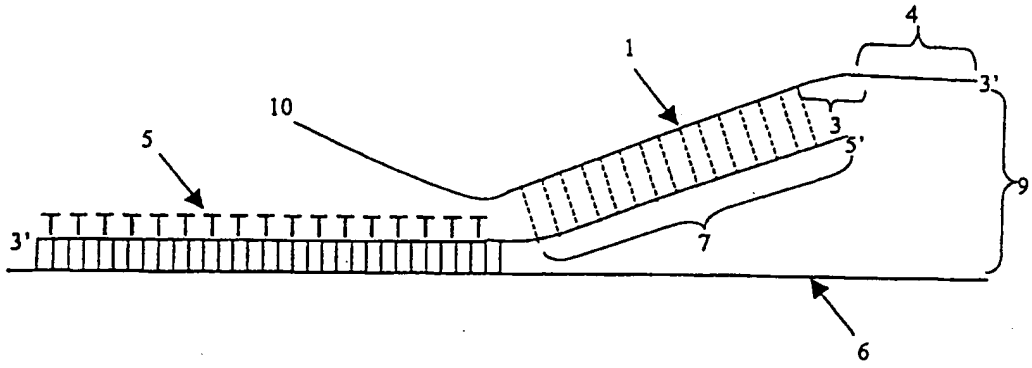
1.


5'
3'
1
2
4
10
3'
17
3

2.


8
5'
7
3'
5
6

3.


4
1
3'
10
3
5
5'
9
7
3'
6

4.

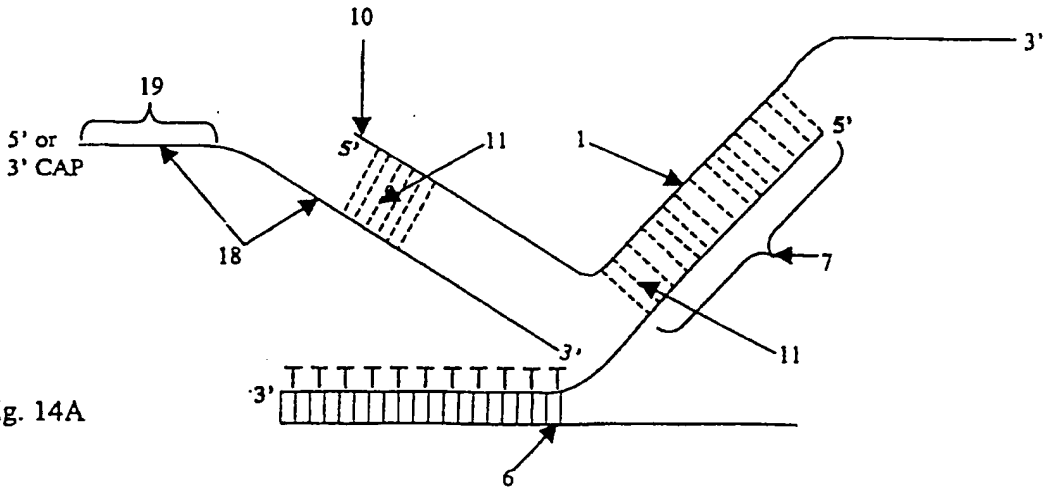
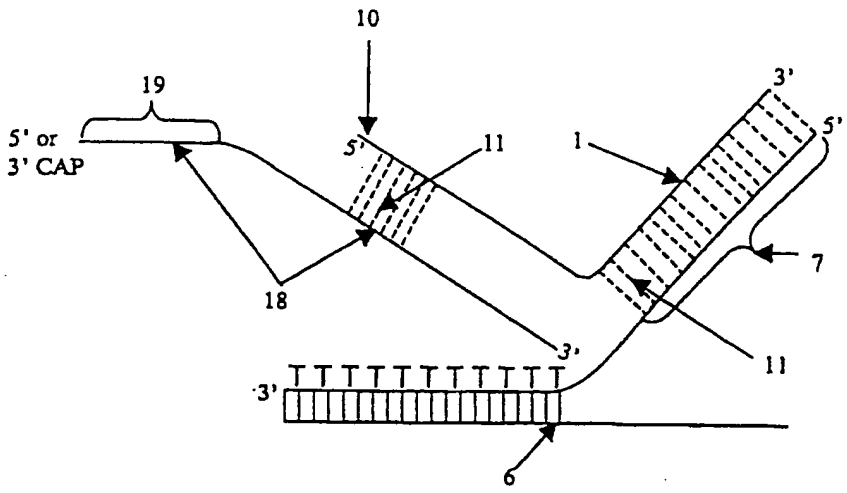

10
3'
19
5' or
3' CAP
5'
11
1
5'
18
7
3'
11
3'
6

Fig. 14A

5.


10
19
5' or
3' CAP
5'
11
1
3'
5'
7
18
3'
11
3'
6

6.

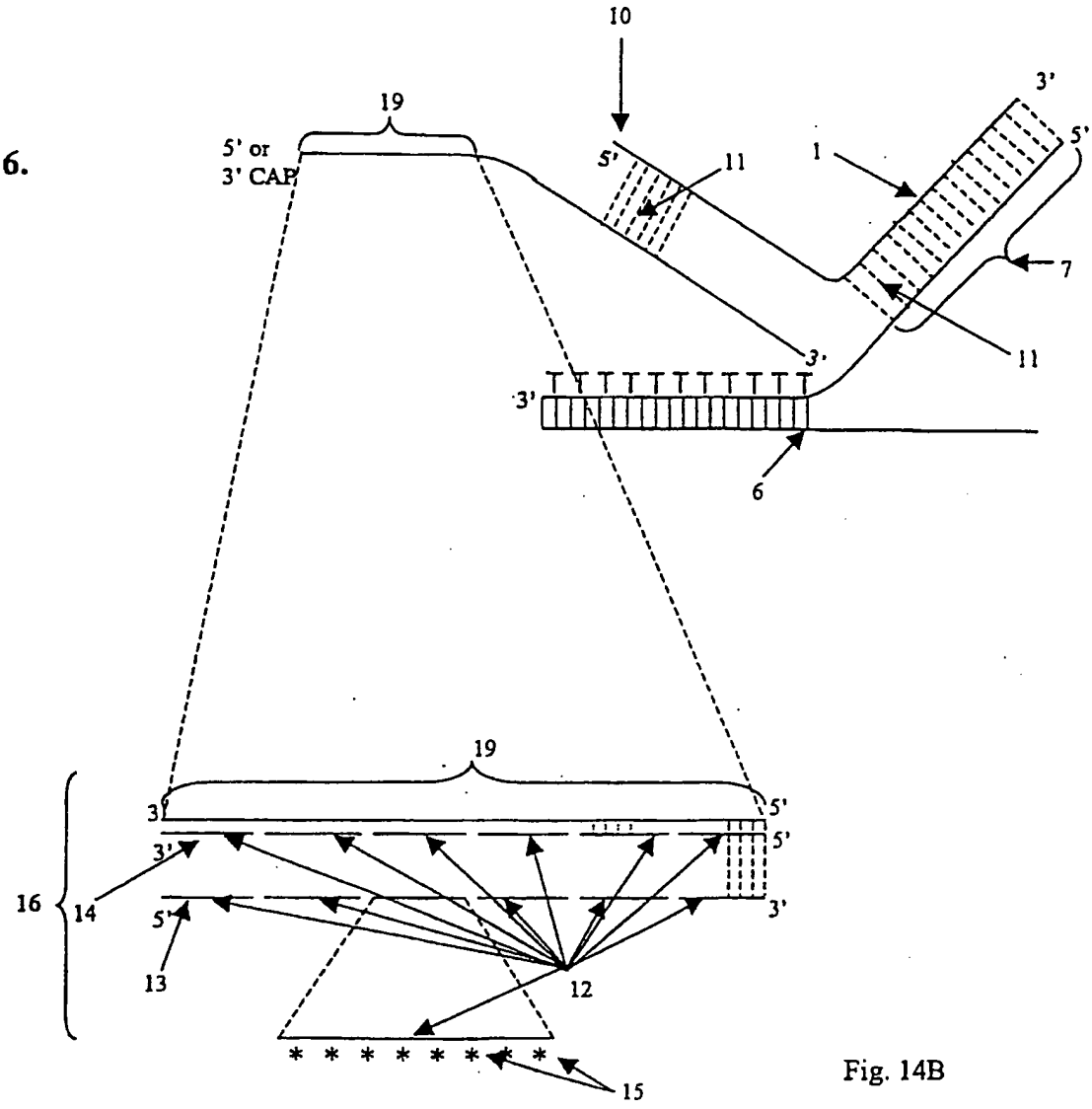

10
19
5' or
3' CAP
5'
11
1
3'
5'
7
3'
11
3'
6
19
3'
5'
16
14
3'
5'
5'
3'
13
12
15

Fig. 14B

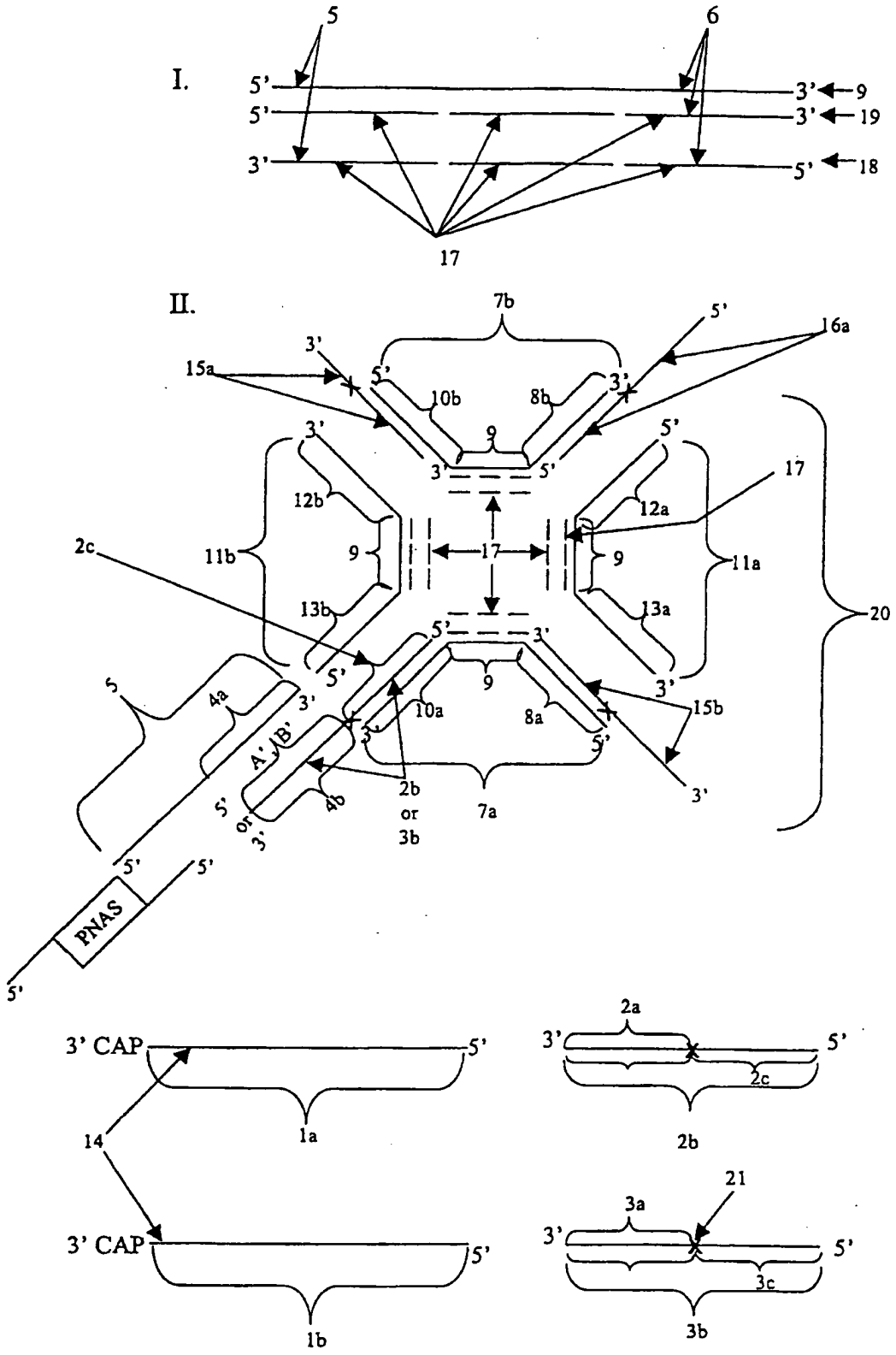

I.
5
6
5'
3'
9
19
18
17
II.
7b
16a
15a
10b
8b
12b
12a
11b
11a
2c
13b
13a
20
4a
A'
B'
4b
2b
or
3b
7a
10a
8a
15b
PNAS
3' CAP
1a
14
1b
2a
2c
2b
21
3a
3c
3b

Fig. 15

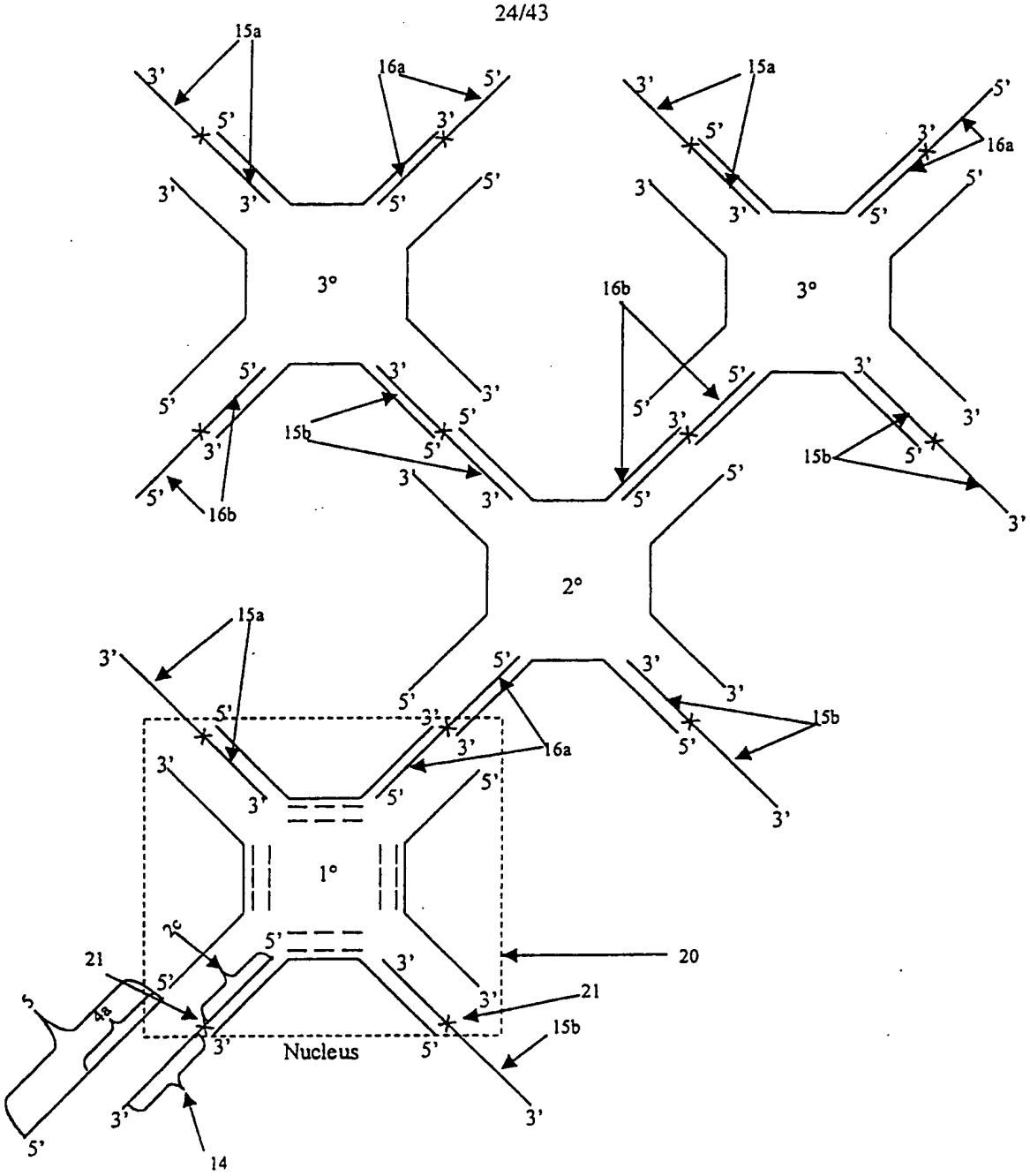


Fig. 16

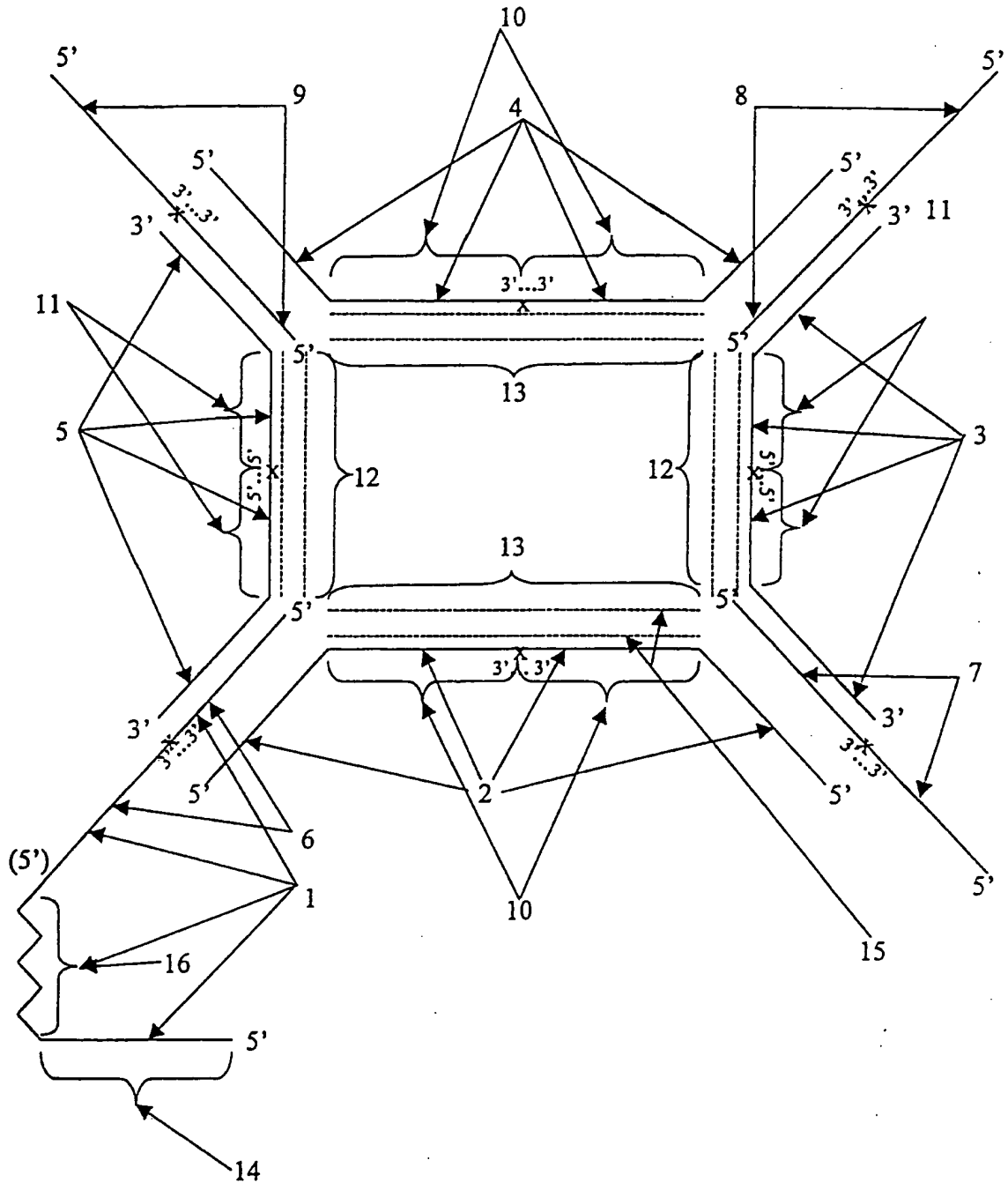

10
5'
9
8
5'
4
5'
5'
3'...3'
3'...3'
3'
3' 11
3'...3'
11
5'
5'
13
5
3
5'...5'
12
12
5'...5'
13
5'
5'
3'
3'...3'
7
3'
3'...3'
5'
2
5'
6
(5')
1
10
15
16
5'
14

Fig. 17

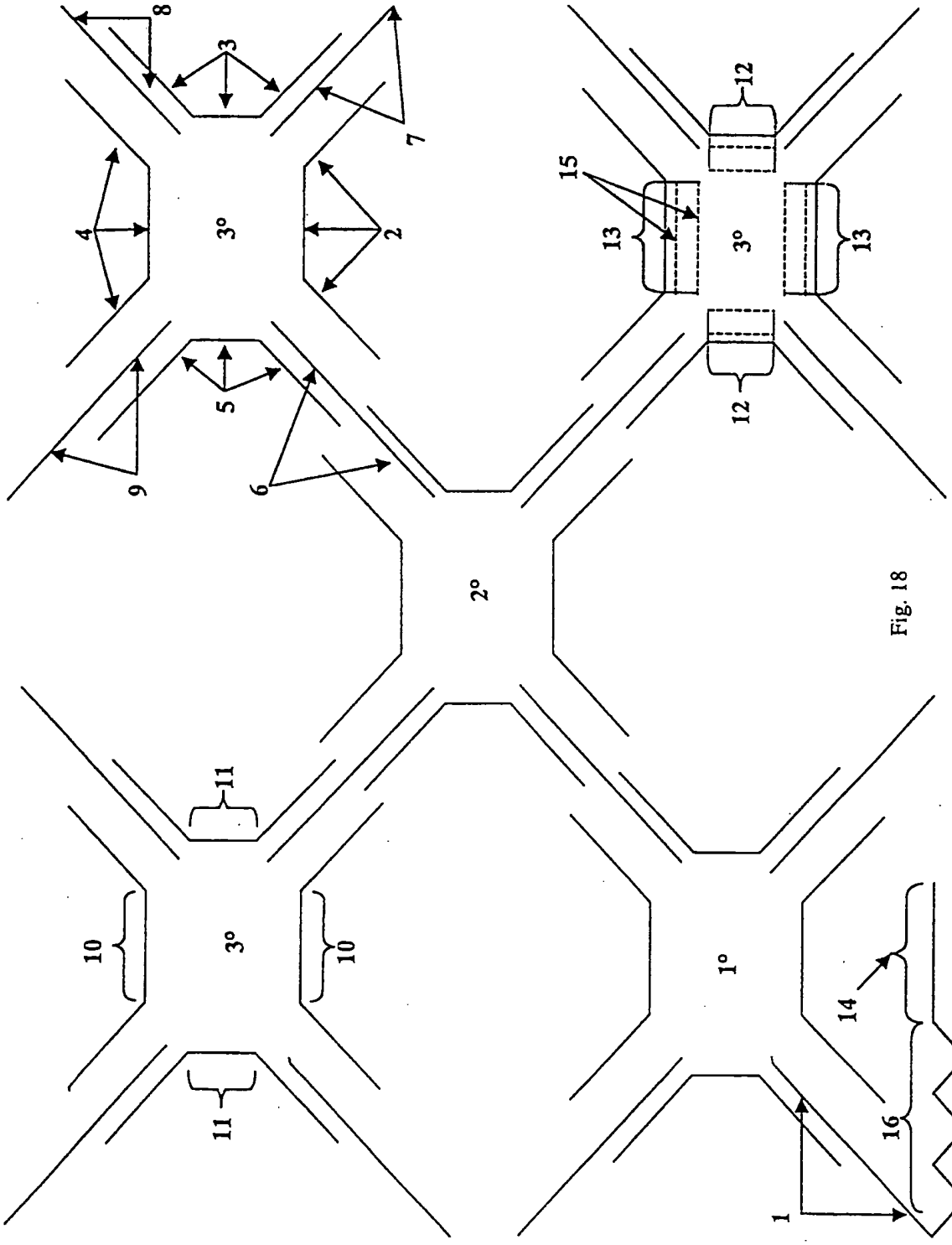


Fig. 18

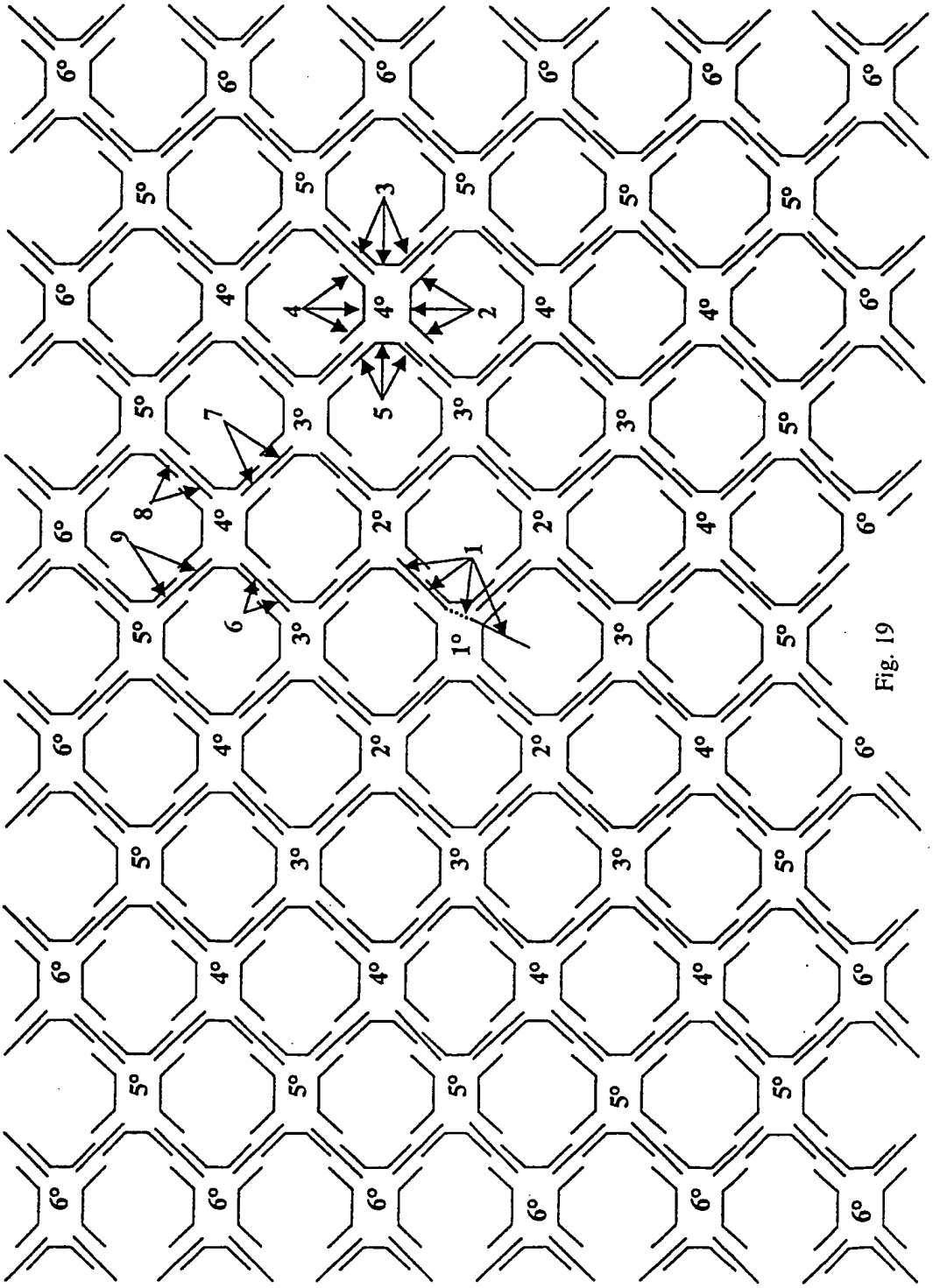
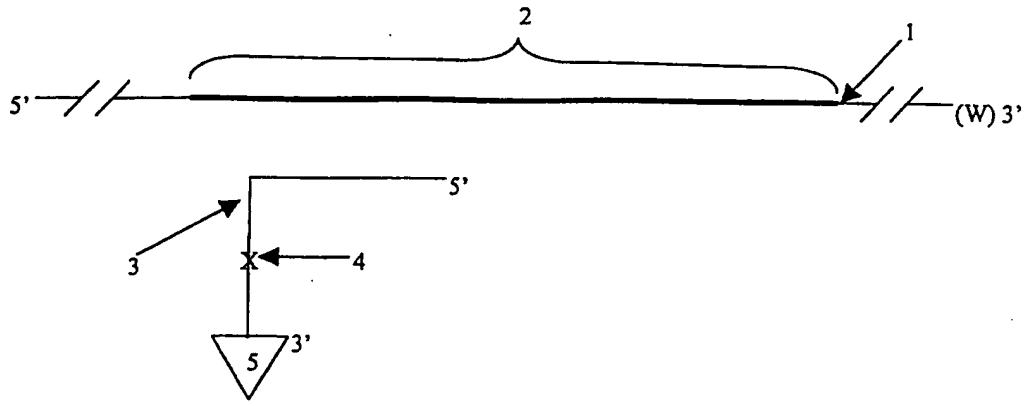


Fig. 19

1.



2.

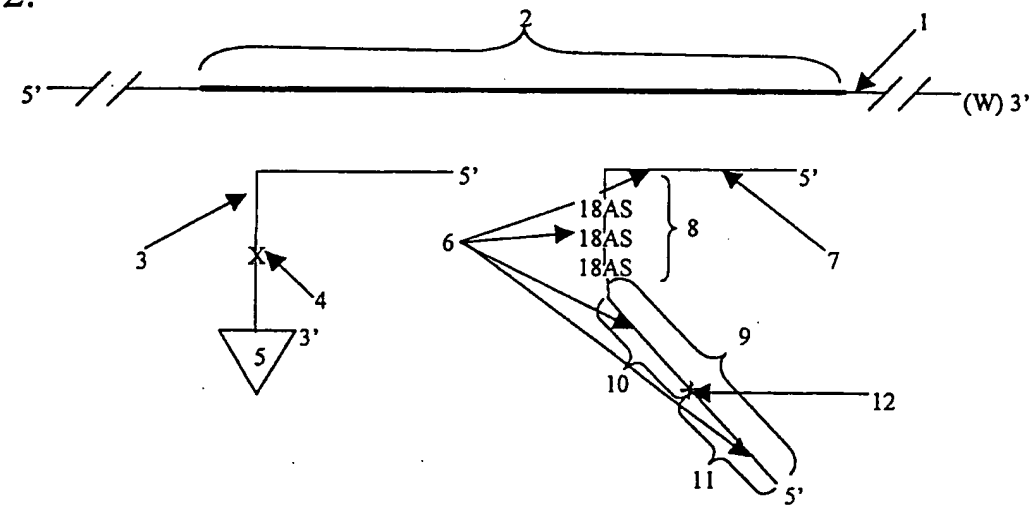
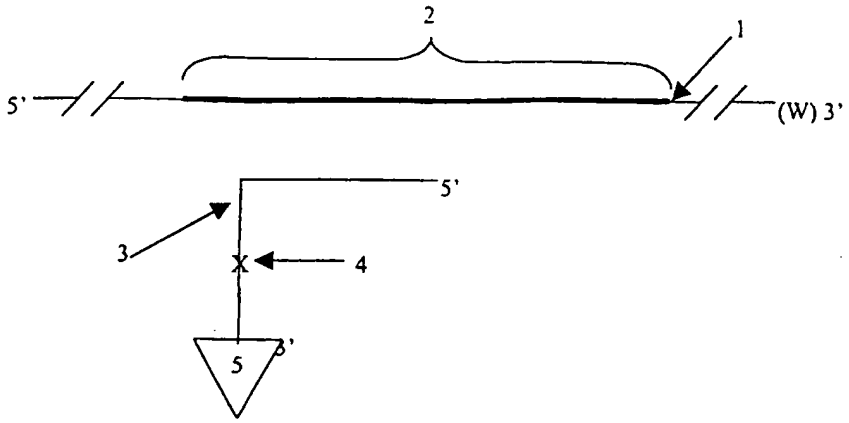
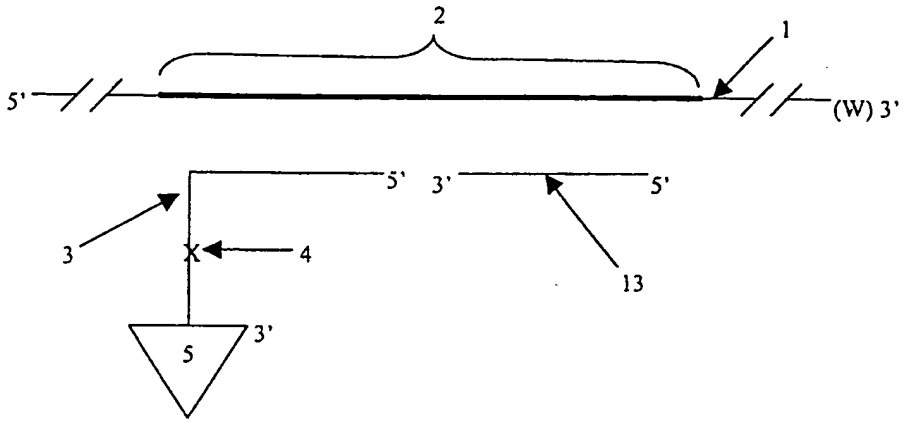


Fig. 20

1.


2
1
5'
(W) 3'
5'
3
X
4
5
3'

2.


2
1
5'
(W) 3'
5'
3'
5'
3
X
4
13
5
3'

3.

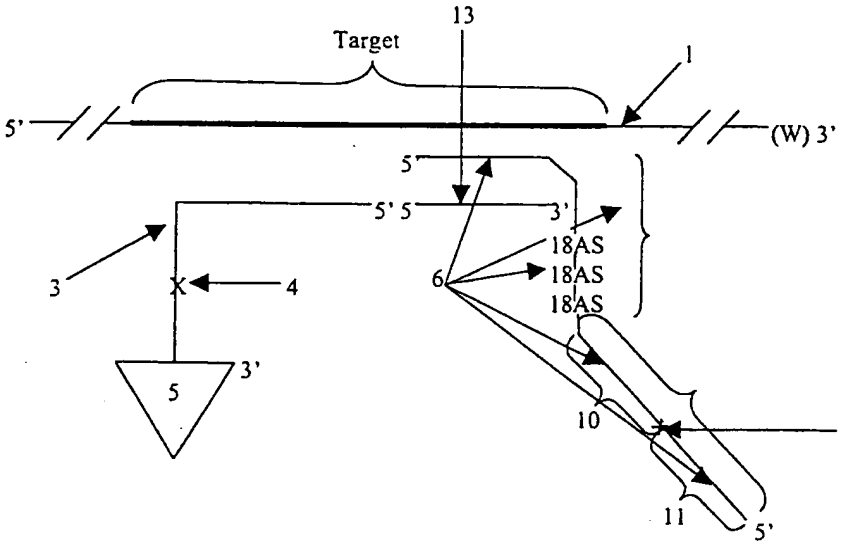
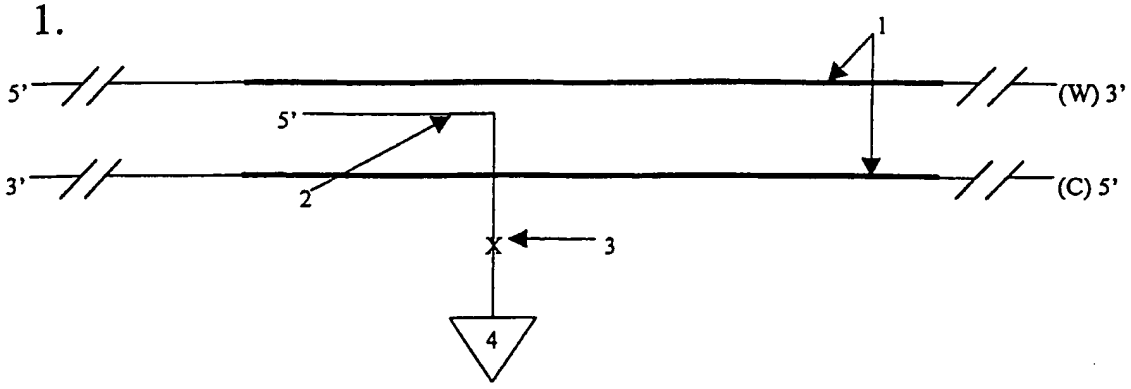

13
Target
1
5'
(W) 3'
5'
5' 5
3'
18AS
18AS
18AS
3
X
4
6
5
3'
10
11
5'

Fig. 21

1.



2.

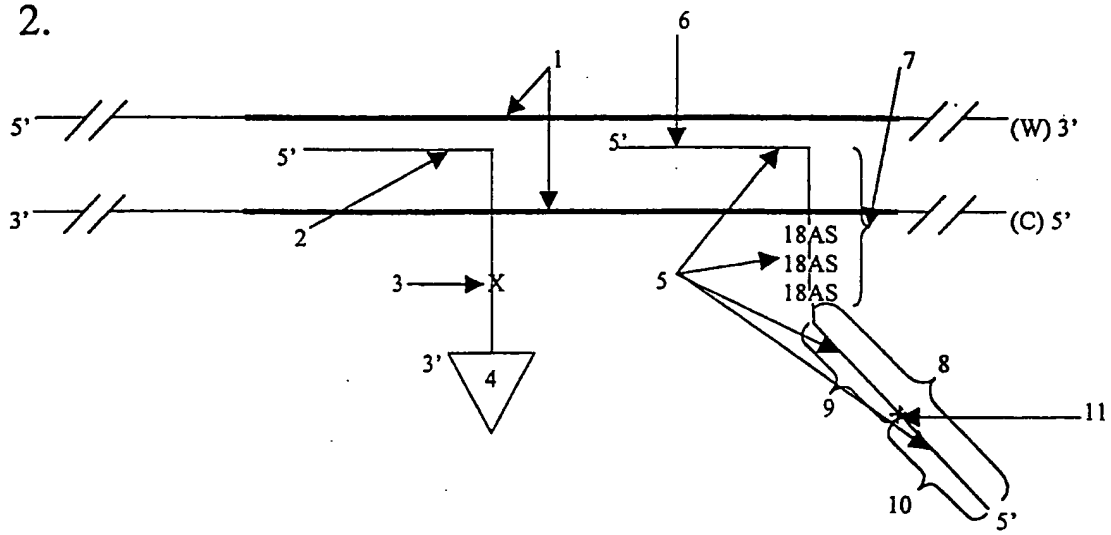
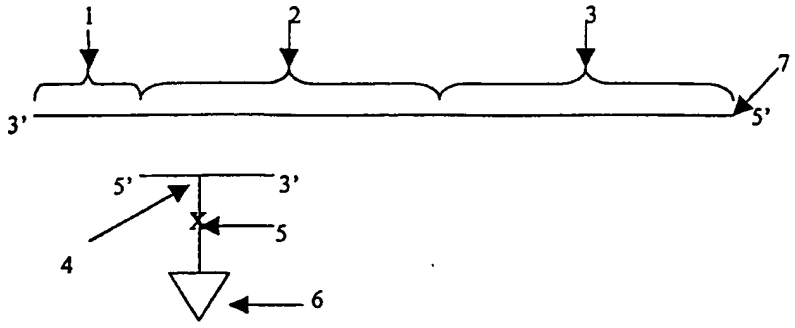


Fig. 22

1.



2.

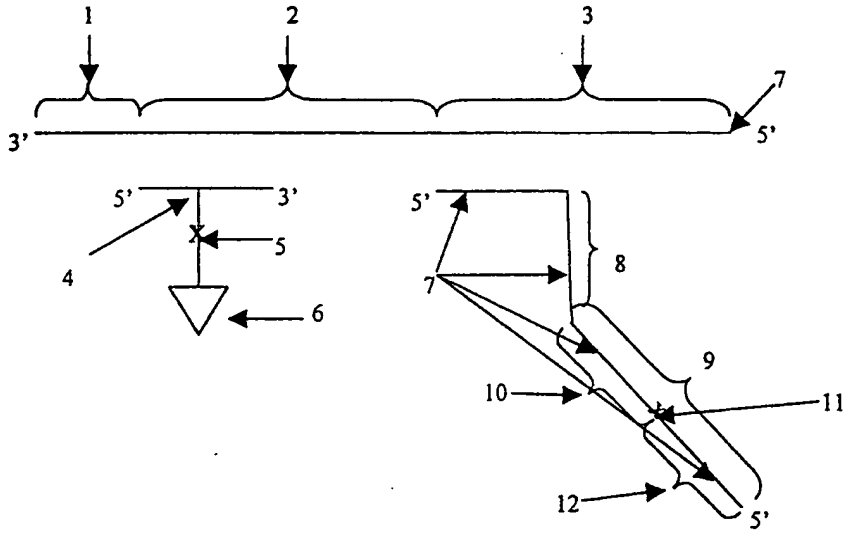
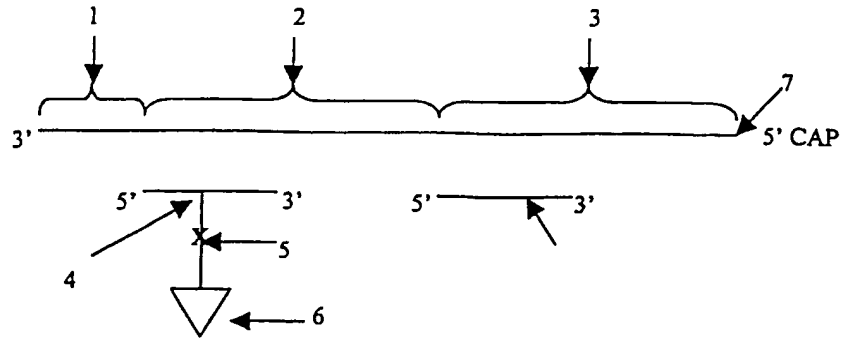


Fig. 23

1.



2.

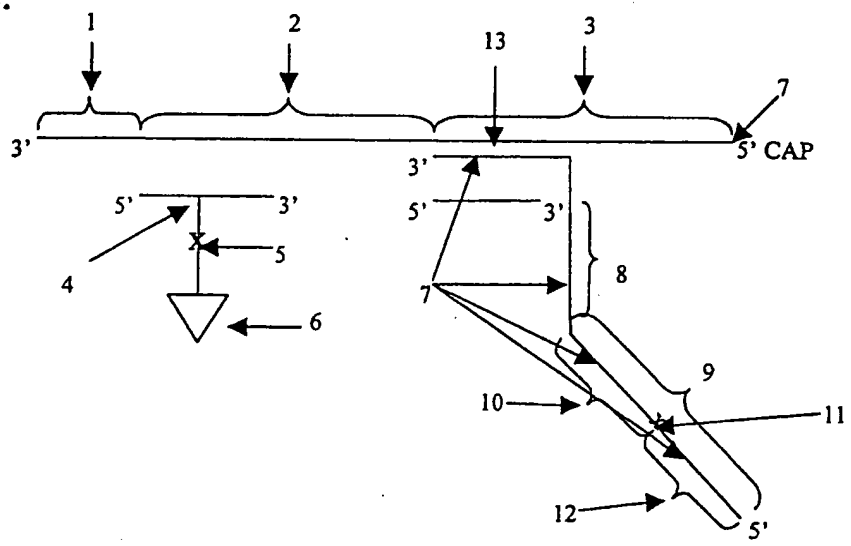


Fig. 24

33/43

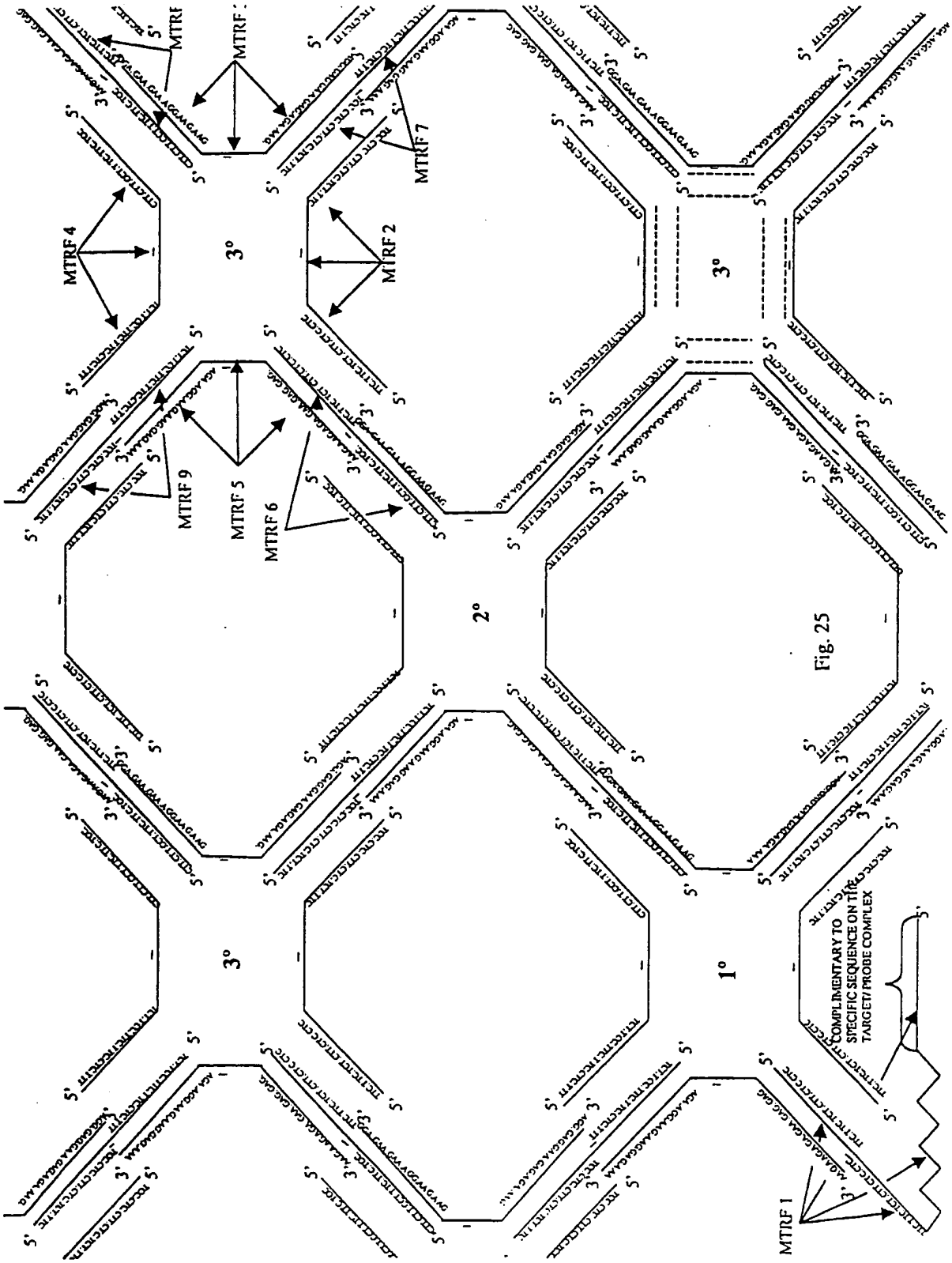

MTRF 4
MTRF 2
MTRF 7
MTRF 9
MTRF 5
MTRF 6
MTRF 1
3°
2°
1°
COMPLIMENTARY TO SPECIFIC SEQUENCE ON TARGET/PROBE COMPLEX

Fig. 25

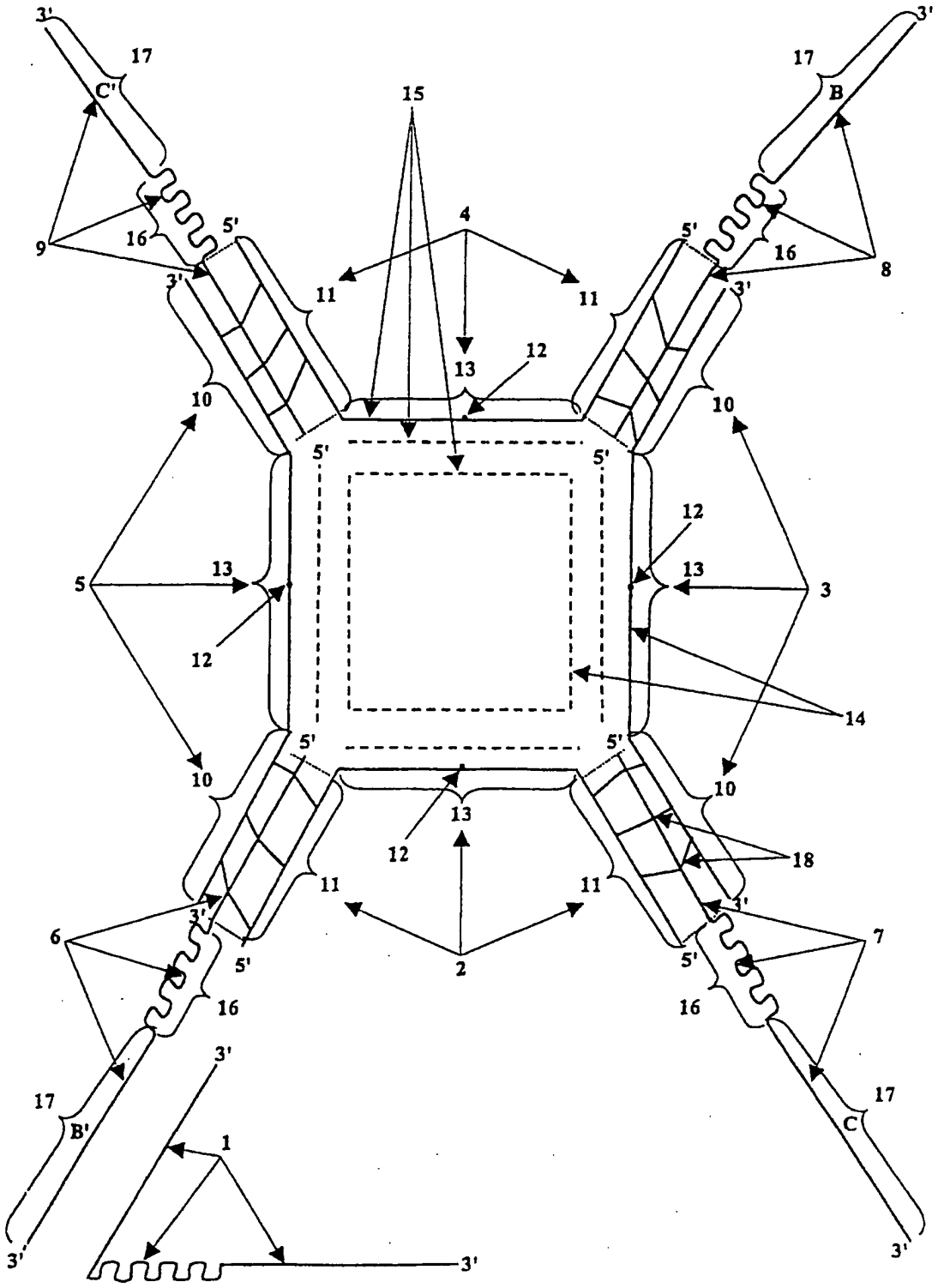

3'
17
C'
15
17
B
3'
4
9
16
5'
5'
16
8
3'
11
11
3'
12
13
10
10
5'
5'
12
13
5
13
3
12
14
5'
5'
10
10
13
12
18
11
11
3'
3'
6
7
5'
5'
2
16
16
3'
17
17
B'
C
1
3'
3'
3'

Fig. 26

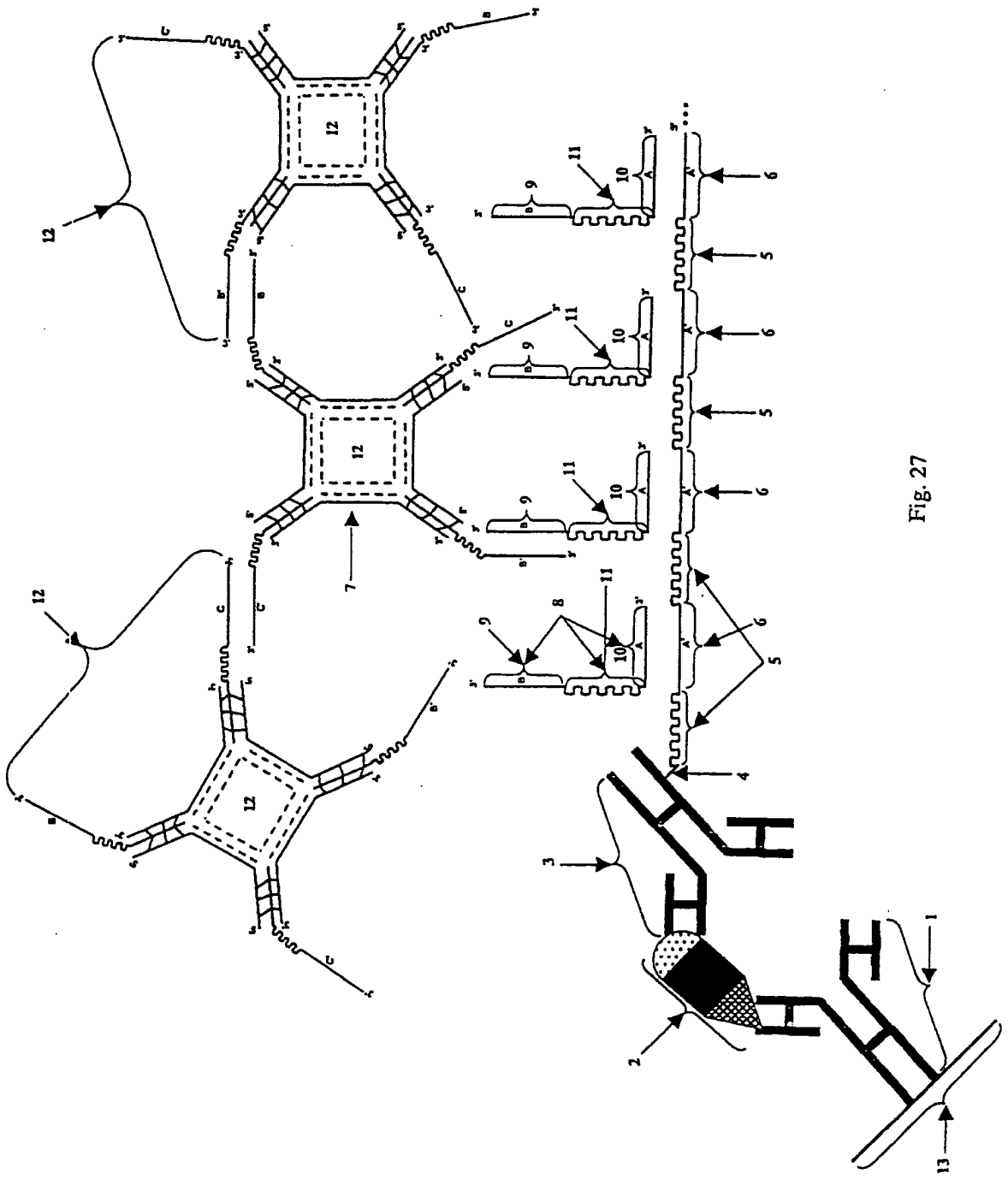


Fig. 27

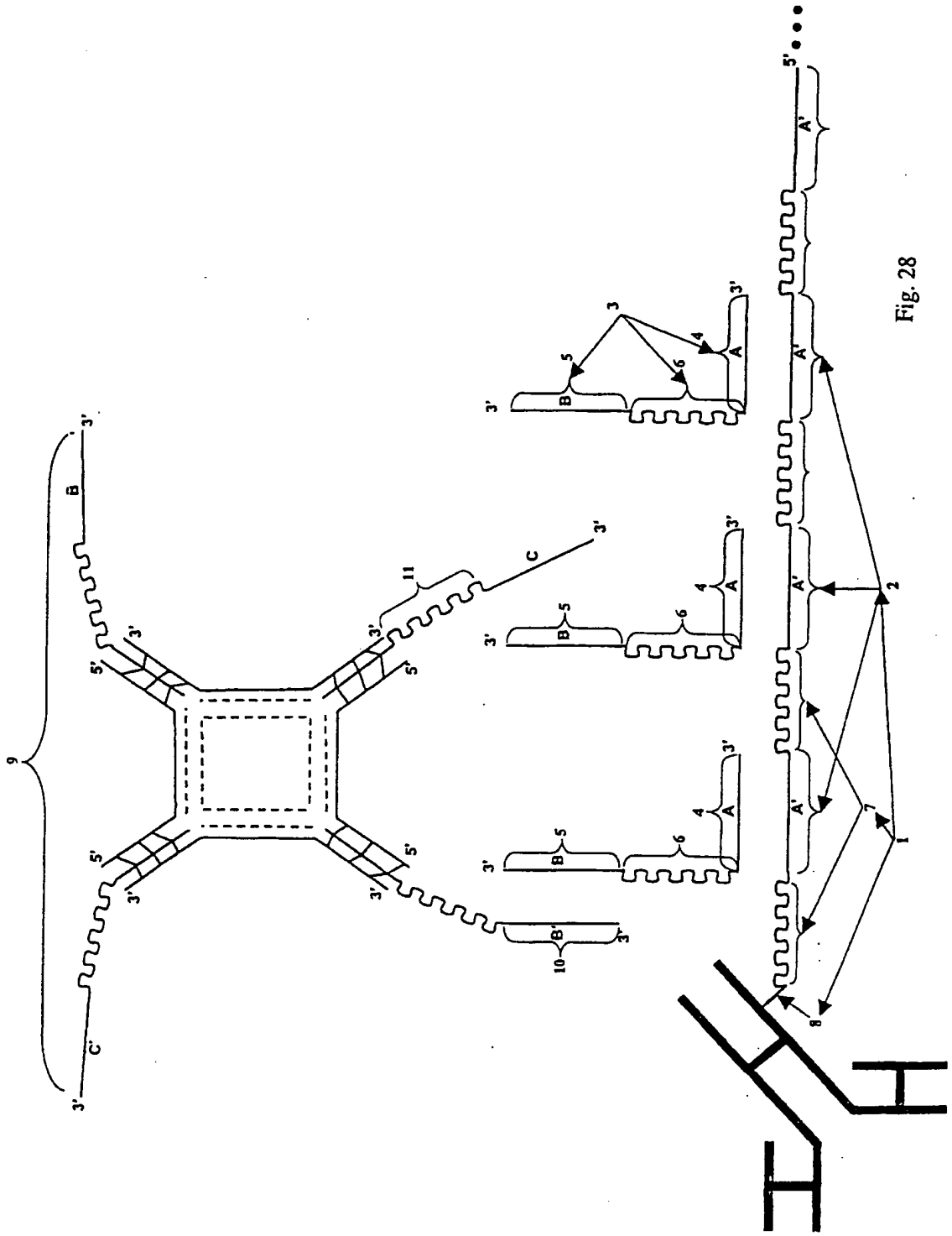


Fig. 28

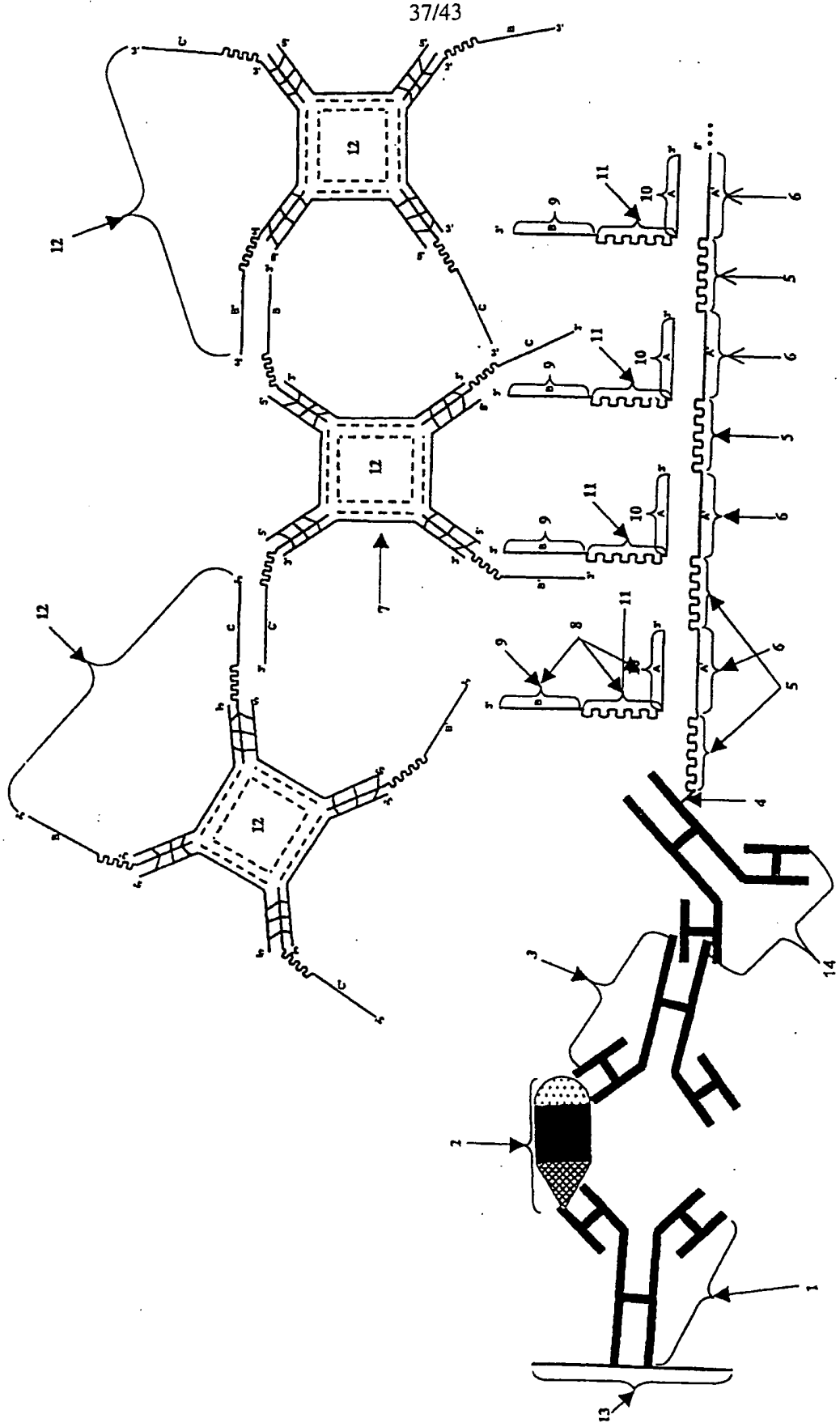
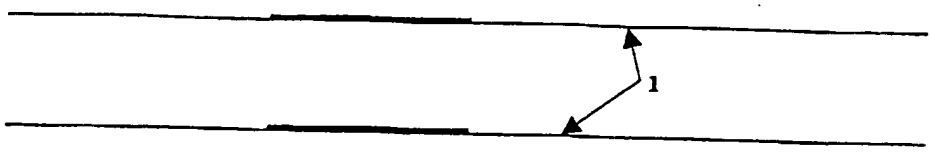
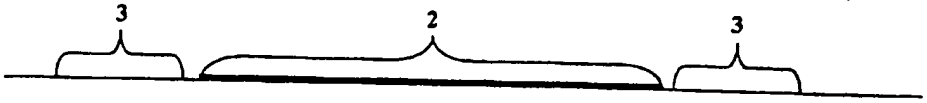


Fig. 29

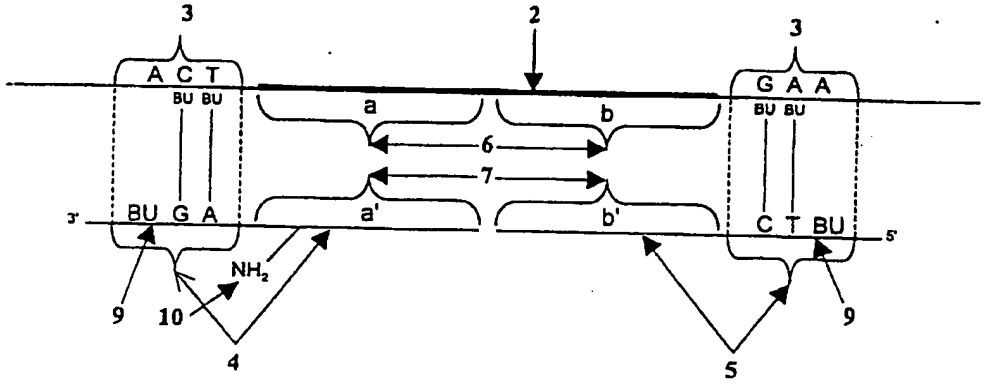
1.


1

2.


3
2
3

3.


3
2
3
A C T
BU BU
G A A
BU BU
a
b
6
7
3'
BU G A
a'
b'
C T BU
5'
NH2
9
10
4
5
9

4.

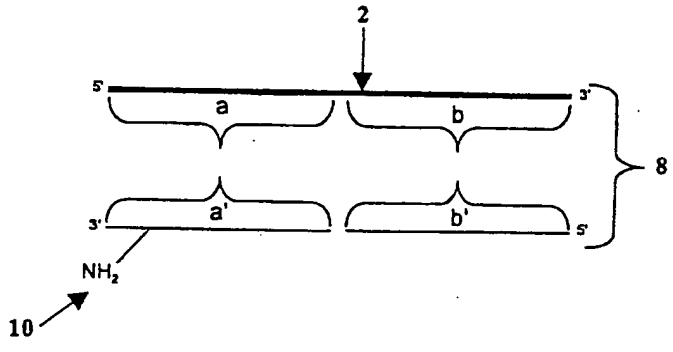

2
5'
3'
a
b
8
3'
a'
b'
5'
NH2
10

Fig. 30

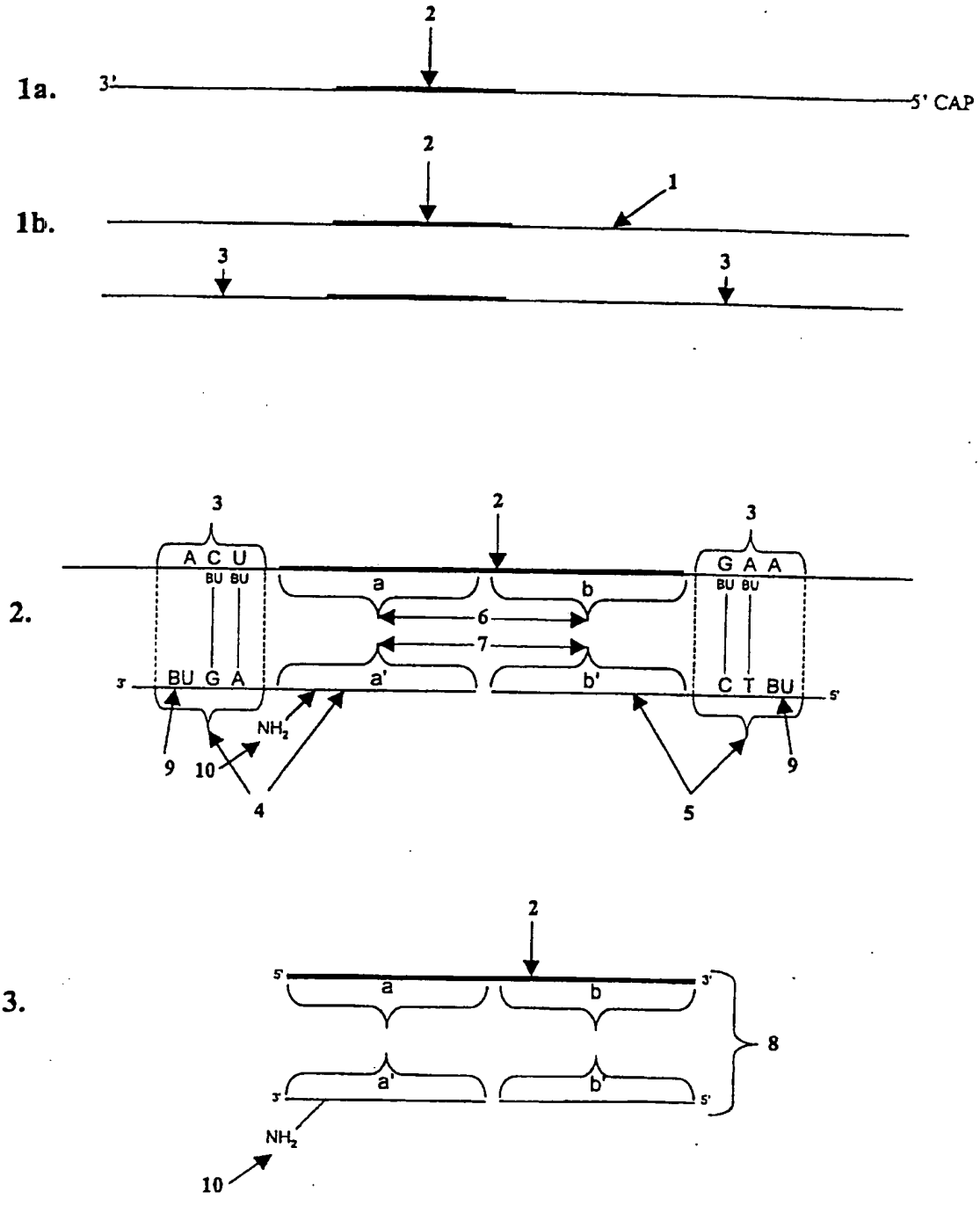
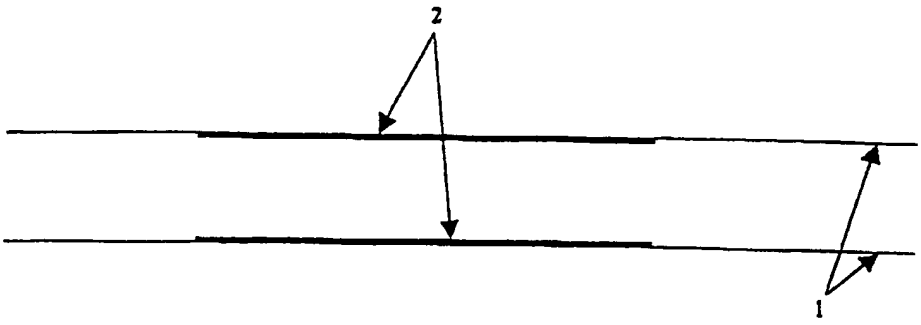
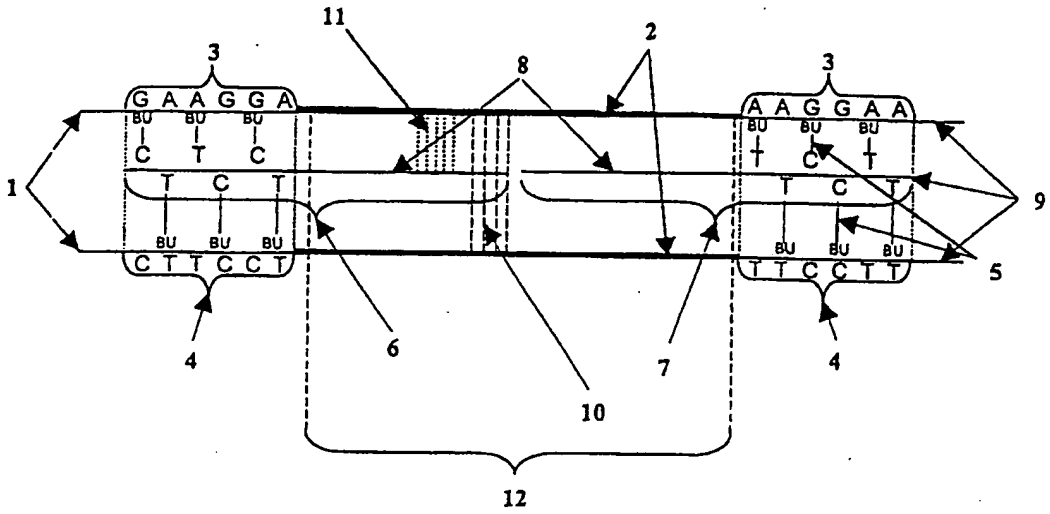

1a.
2
3'
5' CAP
1b.
2
1
3
3
2.
3
2
3
A C U
BU BU
G A A
BU BU
a
b
6
7
3'
BU G A
a'
b'
C T BU
5'
NH2
9
10
4
5
9
3.
2
5'
a
b
3'
8
a'
b'
3'
5'
NH2
10

Fig. 31

1.


2
1

2.


11
3
8
2
3
G A A G G A
A A G G A A
BU
C T C
T C T
BU BU BU
C T T C C T
T T C C T T
1
9
5
4
6
7
4
10
12

3.

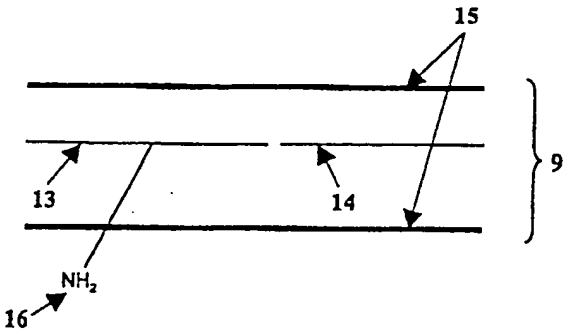
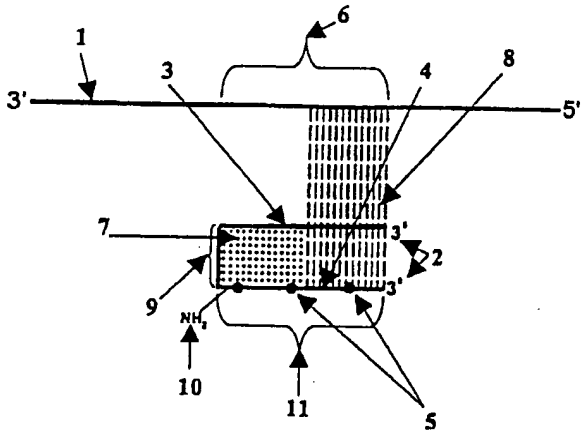

15
13
14
9
NH2
16

Fig. 32

1.


1
6
3
4
8
3'
5'
7
3'
2
3'
9
NH2
10
11
5

2.

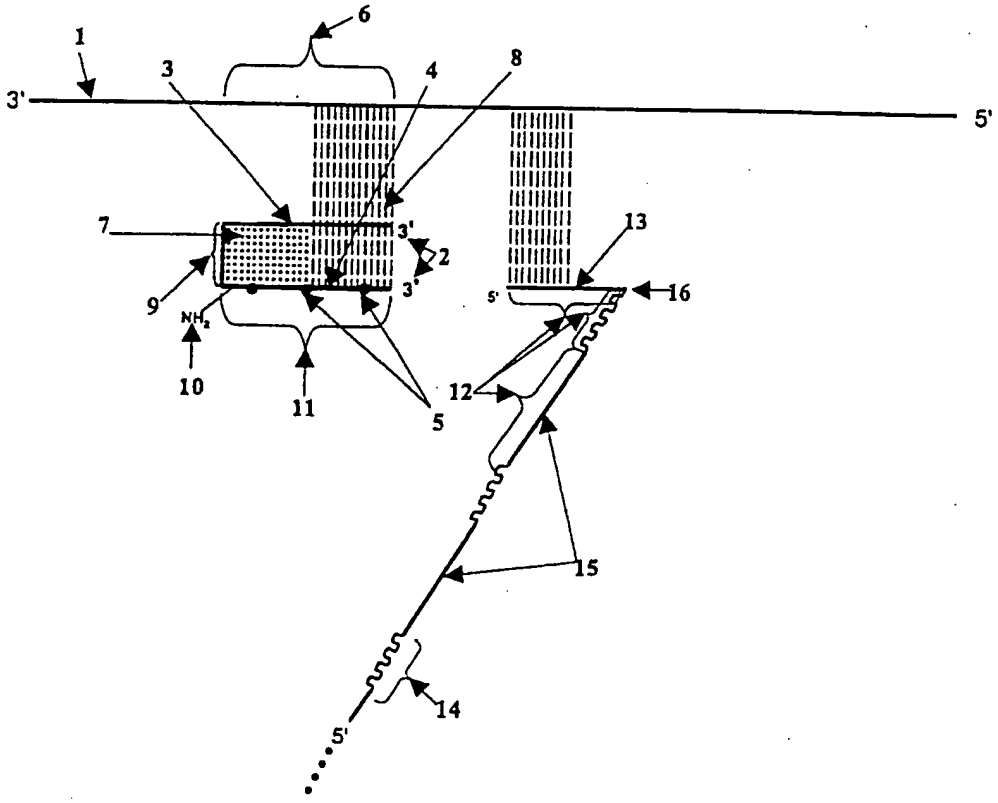

6
1
3
4
8
3'
5'
7
3'
2
3'
13
5'
16
9
NH2
10
11
5
12
15
14
5'

Fig. 33A

3.

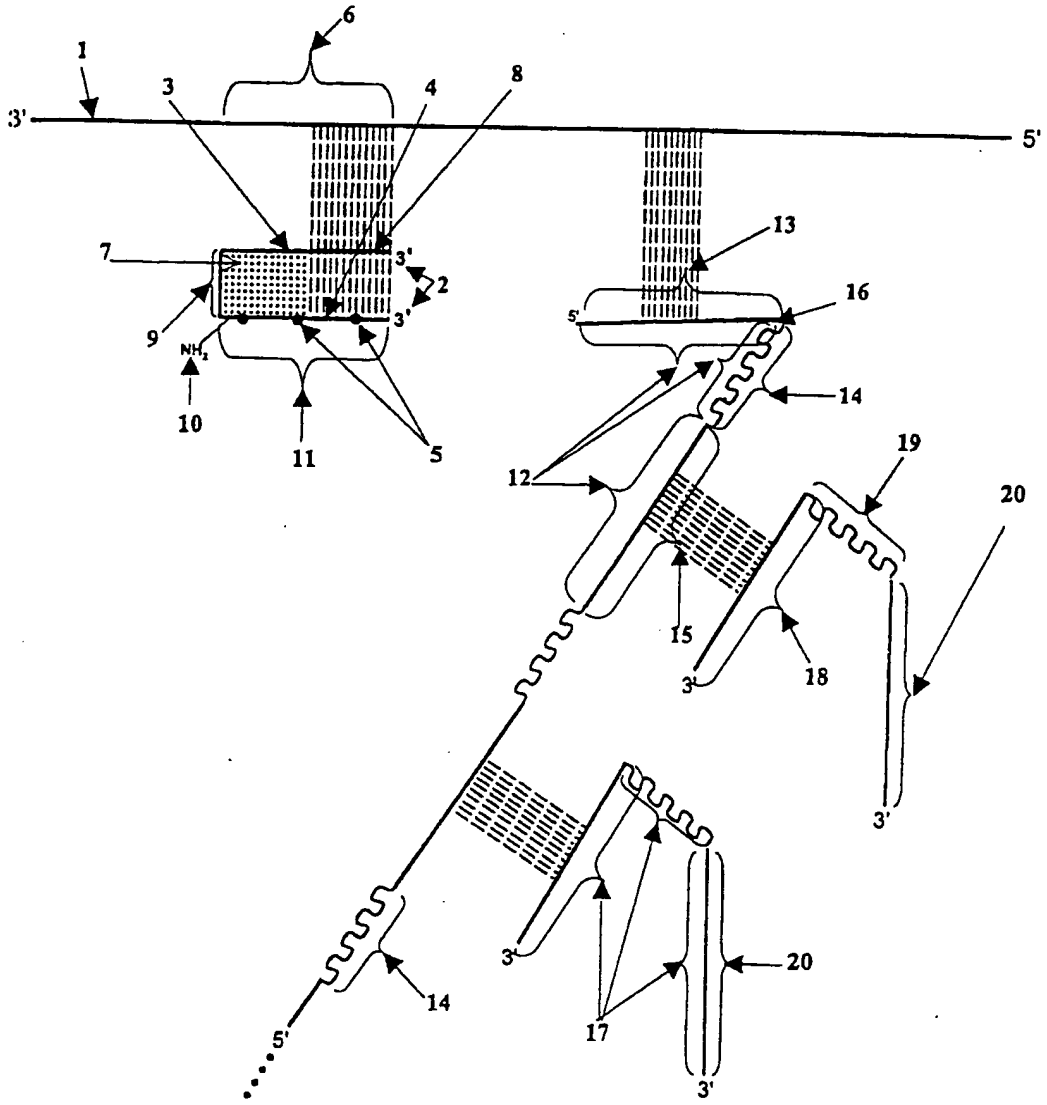
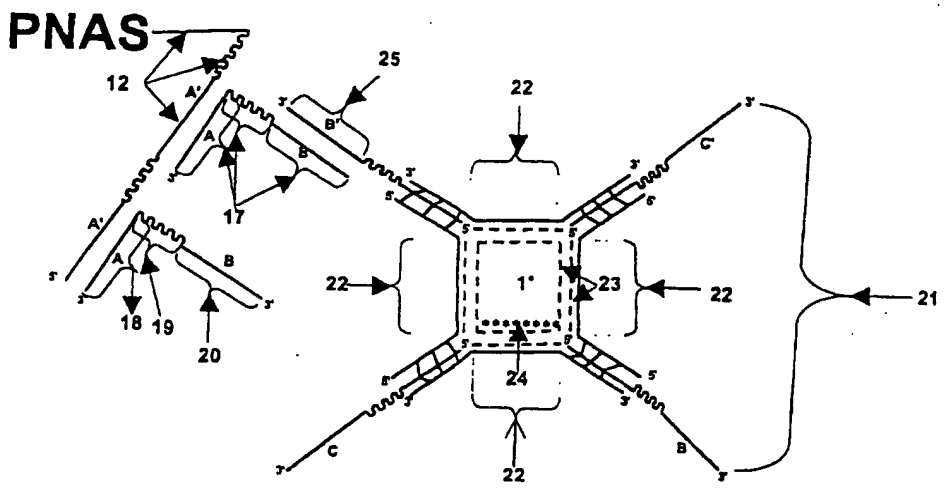


Fig. 33B

4.



5.

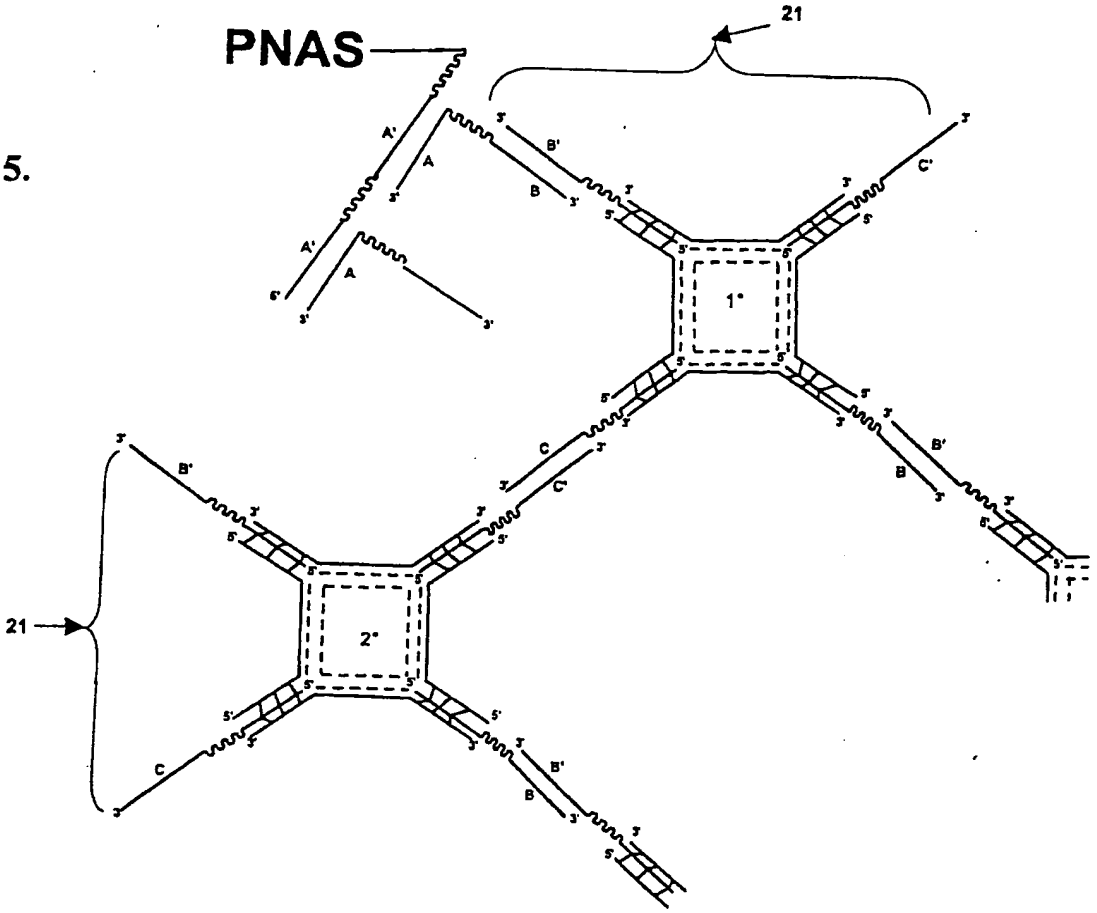


Fig. 33C